PROSTAGLANDIN SIGNALLING AND CONTROL OF MMP PRODUCTION IN HUMAN FETAL MEMBRANES

Nancy J Carr

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Abstract

Premature labour is a major problem in obstetrics and is the leading cause of perinatal morbidity. At present there are no effective diagnostic indicators of preterm birth, and there are no effective treatments for this condition. Current research is therefore focused on understanding the physiology and pathophysiology of the birth process to potentially develop better diagnostic indicators and improve methods of therapeutic management. Prostaglandins, particularly PGE_2 and $PGF_{2\alpha}$ produced within the intrauterine tissues, play a central role in the initiation and progression of human labour. These prostaglandin responses are mediated through a family of seven-transmembrane G-protein coupled receptors, EP1-4 for PGE_2 and FP for $PGF_{2\alpha}$. It is thought that one of the targets of prostaglandin action is the family of matrix metalloproteinases (MMPs). MMPs, particularly the gelatinases MMP-2 and MMP-9, are thought to play a role in rupture of the fetal membranes prior to parturition. The aim of this thesis is to investigate the cellular pathways by which PGE_2 and $PGF_{2\alpha}$ act via their receptors in human fetal membranes and the effect that these responses have on the production and activation of MMP-2 and MMP-9. The JEG3 choriocarcinoma cell-line was used alongside human tissue as a model of chorion trophoblast cells. PCR and immunohistochemistry were used to identify and localise the prostaglandin receptors, EP2, EP4 and FP. These receptors were localised to the amnion epithelium and fibroblast layer, chorion trophoblast and reticular layer, glandular epithelial cells, stromal cells and vascular endothelial cells of the decidua, placental syncytiotrophoblasts, smooth muscle cells of the myometrium and also JEG3 cells. To investigate intracellular signalling, amnion and chorio-decidua were collected immediately after term elective caesarean sections and treated with PGE_2 and $PGF_{2\alpha}$, and selective inhibitors and antagonists, as were the JEG3 cells. Samples were homogenised and assayed for cyclic AMP (cAMP) and analysed for extracellular signal-regulated kinase (ERK) phosphorylation by immunoblotting. PGE_2 elevated cAMP levels over 2-fold after 10 min in amnion and JEG3 cells, with no effect on chorio-decidua. These effects were inhibited by the addition of an EP2 antagonist in amnion and an EP4 antagonist in JEG3 cells. PGE_2 and $PGF_{2\alpha}$ had no effect on phosphorylation of ERK1/2 in amnion or chorio-decidua, though in JEG3 cells, PGE_2 and $PGF_{2\alpha}$ increased it by 9-fold and 2-fold respectively after 10 min. Pre-treatment with an inhibitor of MEK totally inhibited this stimulation, and an inhibitor of PLC and EGFR kinase had no effect. The ERK1/2 phosphorylation stimulations by PGE_2 and $PGF_{2\alpha}$ were inhibited by an EP4 and an FP antagonist, respectively. To investigate the effects of prostaglandins on MMP production and activation, amnion, chorio-decidua and placenta were collected after term elective caesarean sections and stimulated with PGE_2 and $PGF_{2\alpha}$, as were JEG3 cells. Quantitative PCR was carried out to determine mRNA levels of MMP-2 and MMP-9 after this stimulation. Zymography was performed to reveal latent and active forms of MMP-2 and MMP-9, and reverse zymography and Western blotting carried out to quantify TIMP levels. Results show that although there were no major PG-mediated changes in latent or active forms of MMP protein, MMP-2 mRNA production was up-regulated by both PGs in JEG3 cells. These results suggest that prostaglandins are acting via the EP2, EP4 and FP receptors to activate intracellular signalling pathways which up-regulate the production of MMPs. This mechanism could be involved in rupture of the fetal membranes, and the PG receptor antagonists could potentially be used to block this process to prevent preterm rupture of the membranes.

Declaration

Except where due acknowledgement is made by reference, the studies undertaken in this thesis were the unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

Nancy Carr

Presentations Relating to this Thesis

Oral Presentations

Carr NJ, Leask R, Riley SC (2003) Control of tissue remodelling during labour by matrix metalloproteinase-9 and -2. *Joint meeting of the Munro Kerr Society and the Blair Bell Society*, Glasgow Royal Infirmary, UK

Carr NJ, Leask R, Jabbour HN, Riley SC (2004) PGE_2 and $PGF_{2\alpha}$ signalling via EP2, EP4 and FP receptors in the human amnion and chorio-decidua. *Munro Kerr Society Meeting*, Edinburgh Royal Infirmary, UK

Carr NJ (2004) Prostaglandin signalling and control of MMP production in human fetal membranes. *PhD Seminar, Centre for Reproductive Biology, Edinburgh Royal Infirmary, UK*

Poster Presentations

Carr NJ, Leask R, Jabbour HN, Riley SC (2004) PGE_2 and $PGF_{2\alpha}$ signalling via EP2, EP4 and FP receptors in the human amnion and chorio-decidua. *37th Annual meeting for the Society for the Study of Reproduction*, University of British Columbia, Vancouver, Canada

Carr NJ, Jabbour HN, Riley SC (2004) PGE_2 and $PGF_{2\alpha}$ signalling via EP4 and FP receptors in the JEG3 choriocarcinoma cell-line and their effects on MMP-2. *13th Simpson Symposium and VIIth International Conference on the Extracellular Matrix of the Female Reproductive Tract*, Edinburgh Royal Infirmary, UK

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Abbreviations

AA	arachidonic acid
ABC	avidin biotin peroxidase detection system
AC	adenyl cyclase
ADAM	a disintegrin and metalloproteinase
AP	activating protein
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	adenosine-3',5' –cyclic monophosphate
CBP	CREB binding protein
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
COX-1/2	cyclo-oxygenase-1/2
CRE	cAMP response element
CREB	CRE binding protein
CRH	corticotrophin releasing hormone
CRH-BP	CRH binding protein
CRH-R	CRH receptor
CT-PCPE	Inhibitor of PCPE
DAB	3,3' –diaminobenzidine
DAG	diacylglycerol
DAR	donkey anti-rabbit
dH_2O	distilled water
DHEAS	dehydroepiandrosterone sulphate
DMEM	Dulbecco's Modified Eagles Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol

E1	oestrone
E2	oestradiol
E3	oestriol
ECM	extracellular matrix
EDTA	ethylenediaminetetracetic acid
EGF	epidermal growth factor
EGFR	EGF receptor
ELISA	enzyme linked immunosorbent assay
EMMPRIN	extracellular matrix metalloproteinase inducer
EP1-4	PGE receptors 1-4
ER	oestrogen receptor
ERK	extracellular signal regulated kinase
FAK	focal adhesion kinase
FCS	fetal calf serum
FP	PGF receptor
G-6-PDH	glucose-6-phosphate dehydrogenase
GAGs	glycosaminoglycans
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine exchange factor
GPCR	G-protein coupled receptor
GROα	growth regulated oncogene-α
GTP	guanosine triphosphate
hCG	human chorionic gonadotrophin
hr	hour
HRP	horse radish peroxidase
HSD	hydroxysteroid dehydrogenase
IBMX	isobutylmethyl xanthine
ICAM	intercellular adhesion molecule
IGF	insulin-like growth factor

IgG	immunoglobulin
IL-1/2 etc.	interleukin-1/2 etc.
IFN	interferon
IP_3	inositol 1, 4, 5-triphosphate
JNKs	c-Jun NH_2-terminal kinases
LIF	leukaemia inhibitory factor
MAPK	mitogen activated protein kinase
MEK	extracellular regulated kinase kinase
MIC-1	macrophage inhibitory cytokine-1
min	minute
MLCK	myosin light chain kinase
MMP	matrix metalloproteinase
MOX	methyloximating solution
mRNA	messenger RNA
MT-MMP	membrane type MMP
NBF	neutral buffered formalin
NF-κB	nuclear factor kappa beta
NO	nitric oxide
NOS	nitric oxide synthase
NSAIDs	non-steroidal anti-inflammatory drugs
NSB	non-specific binding
onfFN	oncofetal fibronectin
OTR	oxytocin receptor
PAF	platelet-activating factor
PBS	phosphate buffered saline
PCPE	procollagen C-terminal proteinase enhancer
PCR	polymerase chain reaction
PEA	Polyoma enhancer A-binding protein
PG	prostaglandin
PGD_2, E_2 etc.	prostaglandin D_2, E_2 etc.

PGDF	platelet derived growth factor
PGDH	prostaglandin dehydrogenase
PGDS etc.	prostaglandin D synthase etc.
PGI_2	prostacyclin
PGT	prostaglandin transporter
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PMSG	pregnant mare's serum gonadotrophin
PPAR	peroxisome proliferator activated receptor
PROM	premature rupture of the membranes
PPROM	preterm premature rupture of the membranes
PR	progesterone receptor
PTP	protein tyrosine phosphatase
RNA	ribonucleic acid
RPMI	Rosewell Park Memorial Institute
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription-polymerase chain reaction
RU486	mifepristone
s	second
SAM	sheep anti-mouse
SNP	single nucleotide polymorphism
STP	streptavidin peroxidase
TBS	tris buffered saline
TGF	transforming growth factor
TIMP	tissue inhibitor of matrix metalloproteinases
TK	tyrosine kinase
TMPS	triple-membrane-passing-signalling
TNF-α	tumour necrosis factor-α

TXA_2	thromboxin A_2
UAP	uterine activation protein
VEGF	vascular endothelial growth factor
ZAM	zone of altered morphology

Chapter 1: Introduction

1.1 Introduction

An understanding of the regulation of parturition at term would be of great benefit to the diagnosis, management and outcome of preterm birth, a major problem in clinical obstetrics. Preterm birth occurs in around 10 % of all pregnancies and accounts for 70 % of perinatal morbidity. It is defined as birth prior to 37 of the usual 40 weeks gestation period. During pregnancy, the uterus must be maintained in a relative state of quiescence, allowing the fetus to develop. It must then develop coordinated contractility, and the cervix must dilate to allow the passage of the fetus along the birth canal. The transition from pregnancy to parturition results from a complex interplay of maternal and fetal factors. For a successful birth, maturation of the fetal organ systems necessary for survival in the extra-uterine environment must have occurred, therefore communication between the fetus and the stimulus by which uterine activity is increased is necessary. Preterm birth will result if this synchronisation is at fault. In this chapter, literature will be reviewed to give an overview of events which occur at human parturition. Regulation of these events will be discussed with particular attention to the involvement of prostaglandins, cytokines and matrix metalloproteinases.

1.2 Parturition

Parturition is the physiological process by which the fetus, placenta and fetal membranes are expelled from the uterus into the extra-uterine environment. Labour is the sequence of involuntary uterine contractions that result in dilation of the cervix and delivery of the fetus, placenta and fetal membranes. The first stage of labour begins with progressive dilation of the cervix. The second stage begins when the cervix is fully dilated and ends with delivery of the baby. The third stage begins when the baby is born and ends when the placenta and fetal membranes are expelled. During this stage, retraction of the uterus and manual compression of the abdomen reduce the area of placental attachment. A hematoma (mass of blood) forms, which separates the placenta and the fetal membranes from the uterine wall. The fourth stage follows this and involves myometrial

contractions to constrict the arteries that formerly supplied blood to the intervillous space of the placenta. These contractions prevent excess uterine bleeding and return the uterus to its normal size (uterus involution) (Moore & Persaud 1998). A complex balance of fetal and maternal factors is necessary to facilitate all the processes involved in labour. In this section, the structure of the pregnant uterus and its components will be discussed, followed by many of the different events which occur during labour.

1.2.1 Structure of the Pregnant Uterus

The structure of the pregnant uterus is shown in *Figure 1.1*. It consists of maternal myometrium and decidua, and the fetal membranes and placenta which are genetically identical to the fetus.

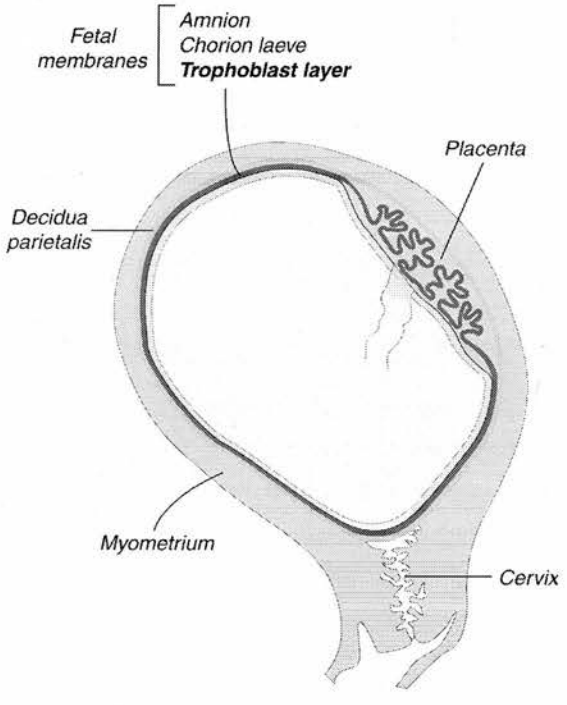


Figure 1.1: A pregnant uterus.

1.2.1.1 Fetal membranes

Fetal membranes consist of amnion and chorion (*Figure 1.2*) (Bourne 1960). The avascular amnion comprises an inner epithelial layer which is in direct contact with amniotic fluid, a basement membrane, a compact layer which varies greatly in thickness, a fibroblast layer consisting of dispersed mesenchymal cells, a spongy layer rich in proteoglycans and an outer reticular layer, underlying the chorion. The chorion is composed of a fibroblast and a reticular layer, basement membrane, and its primary constituent, trophoblast, which is adjacent and adherent to decidua, and continuous with the placenta. Fetal membranes release many autocrine, paracrine and potentially endocrine mediators including prostaglandins, cytokines, steroids, peptides, MMPs and TIMPs (Challis et al 2000). Although amnion derived prostaglandins increase at labour (Bennett et al 1992, Skinner & Challis 1985), whether significant quantities gain access to the myometrium is not certain due to the high concentration of prostaglandin dehydrogenase (PGDH – see section 1.2.3) in the chorion (Cheung & Challis 1989).

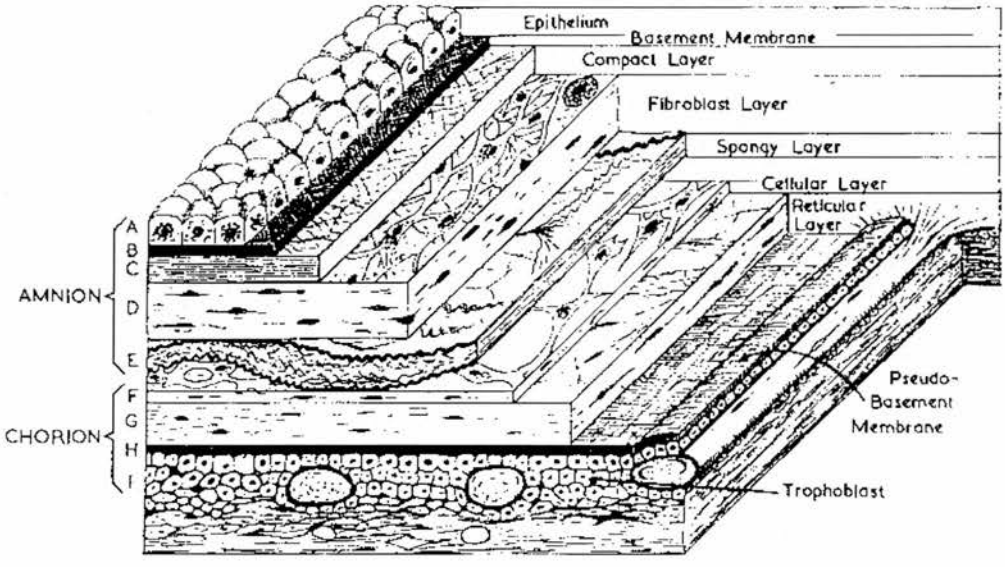


Figure 1.2: Diagram of a section through human amnion and chorion (Bourne 1960).

The major components of these fetal membranes are the cells, and the extracellular matrix (ECM). The cells are responsible for synthesis, degradation and turnover of the ECM, which in turn influences the functions of the cellular component during pregnancy. The fetal membranes require sufficient strength to withstand stretching to approximately double their size at term (Parry-Jones & Priya 1976) and also fetal movements (Patrick et al 1982). But they must also be able to rupture at term, and a build up of normal elastic tissue may prevent this. Thus, it requires a unique structural arrangement which is adaptable to changes in the uterine contents. The major tensile strength is due to the presence of interstitial collagens, I, II, and III, together with smaller amounts of types V, VI and VII in the amnion compact layer (Malak et al 1993). The basement is mainly collagen IV, which provides a scaffold for assembling other basement components such as laminin, entacin and heparin sulphate proteoglycan. Collagen IV is also associated with the basal lamina of the amnion epithelium and the basement membrane of the chorion. Type VII collagen provides additional attachments linking the basal lamina of the amnion epithelium to the ECM underlying this in the form of anchor fibrils (Keene et al 1987). Collagen XIV is also present in the ECM of the fetal membranes, and the decidua. Minor collagens such as this may be important in mechanical stability of the tissue and provide resilience for stretching (Bryant-Greenwood 1998). Type IV collagen is associated with the chorion trophoblast cells (Aplin & Campbell 1985, Malak et al 1993). This may be important for attachment to decidua, as at parturition, separation within the decidual striae occurs, leaving some decidua (capsularis and parietalis) attached to the chorion (Bryant-Greenwood 1998).

Besides collagens, other components are present in the ECM. Elastin has been identified and expressed in the fetal membranes (Hieber et al 1997). This explains the elastic nature of the fetal membranes. The thickness of the elastin fibres in the fetal membranes is relatively thin compared to that of the uterus (Leppert & Yu 1991). The thinness of the fibres may represent a compromise between requirement for elasticity, and a necessity for normal rupture at term. Data suggests a high cross-linking of elastin in the fetal membranes with the enzyme, lysyl oxidase being responsible for this (Kagan &

Trackman 1991, Pinnell & Martin 1968). It is possible that a lack or reduction in elastin in the fetal membranes could play a role in their premature rupture Microfibrils, composed mainly of fibrillin have been found to be particularly abundant in the fibroblast and reticular layers of the amnion and also chorion trophoblast (Malak & Bell 1994a). These are thought to contribute to the elasticity of the fetal membranes, infact it is not clear if microfibrils alone, in the absence of elastin, could provide enough of the required elasticity to the fetal membranes. Fibronectins are synthesised by a wide variety of cells and are organised into ECM. The whole family arises from alternative splicing of a single gene. They have multiple binding domains, capable of adhering to many cells, hence them being termed 'glue-like' (Feinberg et al 1991). Oncofetal fibronectin (onfFN) is present wherever trophoblasts make contact with extraplacental ECM, and can potentially be used to predict preterm labour (see section 1.23). Laminins are a major component of basement membranes and are formed from several subunits linked by disulphide bonds forming a cross-like structure (Timpl et al 1979). They have also been identified in the amnion epithelial layer (Alitalo et al 1980). The precise role of laminins in the fetal membranes is unclear but it is thought hat they may have a role in adhesion, migration and differentiation of invading trophoblasts in early pregnancy. There are also a number of other extracellular matrix components in the fetal membranes including integrins, proteoglycans, decorin and plasminogen (Bryant-Greenwood 1998).

While ECM is crucial for structural and biomechanical properties of the fetal membranes, cell-matrix interactions are very important within the fetal membranes for regulating cell actions and behaviour. The interactions allow the cells and matrix to respond to the changing needs of the tissue. Cell-matrix contacts can influence gene transcription within cells, for example the MMPs which can then act on the ECM. Mechanical changes in the ECM can influence autocrine/paracrine responses in the cells and the ECM. The interactions are particularly important during rupture of the fetal membranes (section 1.2.2.1).

1.2.1.2 Amniotic Fluid

Amniotic fluid is 98-99% water. The chemical composition of its substances varies with gestational age (Modena & Fieni 2004). During the third trimester of pregnancy, total solute concentration in amniotic fluid falls, while the concentration of urea, uric acid and creatine increase. Amniotic fluid is in a dynamic state, complete exchange of its water component within the fetus taking place approximately every 3 hours. Exchange of water and solutes within the fetus occurs via gastrointestinal, urinary and respiratory tracts and, until around week 20, the skin of the fetus is not keratinised, so amniotic fluid exchanges freely with fetal extracellular fluid. Also, the amnion epithelium is a route of exchange. The primary function of amniotic fluid is to protect the fetus from external trauma. It also acts to store waste products and inflammatory mediators. Prior to, and during labour, concentration of cytokines, IL-1, IL-6 and IL-8 (Halgunset et al 1994, Laham et al 1993, Romero et al 1990a, Romero et al 1989a, Romero et al 1992a) and prostaglandins, particularly PGE_2 and $PGF_{2\alpha}$ (Mitchell et al 1979, Romero et al 1994, Romero et al 1989b) increase significantly within amniotic fluid. Unpublised data in the laboratory has also shown that production of MMP-9 and activation of MMP-2 is significantly higher in amniotic fluid at spontaneous labour compared to elective caesarean section at term. This is supported by further data showing MMP-2 and MMP-9 levels increasing with labour (Fortunato et al 1999, Romero et al 2002) and will be discussed in more detail in Chapter 5.

1.2.1.3 Decidua

Under the influence of progesterone, endometrial stromal cells enlarge, proliferate and store glycogen, converting the endometrial lining of the non-pregnant uterus into the decidua of pregnancy. During the first half of the pregnancy, decidual stroma comprises mainly large granular lymphocytes and trophoblast associated macrophages with some T lymphocytes and very few B lymphocytes (Chard & Lilford 1997, Starkey et al 1988, Vince & Johnson 2000). As pregnancy progresses, numbers of large granular lymphocytes decline in number resulting in term decidua being much less distinct and lined with flattened epithelium. T cells and CD16+ lymphocytes increase in term

deciduas, whilst CD56+ lymphocytes decrease, possibly reflecting immunological adaptations of the deciduas during pregnancy (Vargas et al 1993) It is thought that at term, cytokine production by tissues at the feto-maternal interface, including the decidua, favours the Th-1 type, such as IL-1 and IL-6 (see section 1.2.2.4). Alongside cytokines, many factors are synthesised within the decidua including hormones such as prolactin and relaxin, and prostaglandins, which, at parturition, are likely to act in a paracrine manner with those released by fetal membranes (Dudley et al 1992).

Decidua is divided into three regions in relation to the developing fetus. Decidua basalis underlies the conceptus at the implantation site, is adjacent to the chorion and is involved in formation of the maternal side of the placenta. The decidua capsularis lies over the surface of the conceptus and the decidua parietalis lines the remainder of the uterus (Ritchie 1995).

1.2.1.4 Placenta

Soon after implantation, extremely rapid trophoblast mitosis and differentiation begins, with invasion of the maternal uterine stroma. By day 21 after ovulation, the chorionic villus, the definitive functional and structural unit of the placenta, is already established. The trophoblast can differentiate into villous and extravillous trophoblast. In the villous trophoblast, the cytotrophoblasts of the floating villi, which are found in the intervillous space, remain attached to the villous basement membrane. They form a monolayer of epithelial cells that proliferate and differentiate by fusion to form a syncytiotrophoblast which covers the entire villi surface. The syncytiotrophoblast is multifunctional, but mainly works in absorption, exchange, and specific hormone functions. The extravillous trophoblast has cytotrophoblast cells of anchoring villi in contact with the uterine wall. These proliferate and invade the uterus, leading to complete remodelling of the spinal artery walls, with the muscle layer disappearing and endothelial cells being replaced by trophoblasts (Evain-Brion & Malassine 2003). A diagram of the structure of the placenta at mid-gestation is shown in *Figure 1.3*. The placenta has many functions including mediating exchange of gases and nutrients, excreting waste products, secreting many

hormones, including progesterone for the maintenance of pregnancy, other steroids and cytokines and contributing a major role in maintaining the feto-maternal immunological interface and the fetus as an allograft.

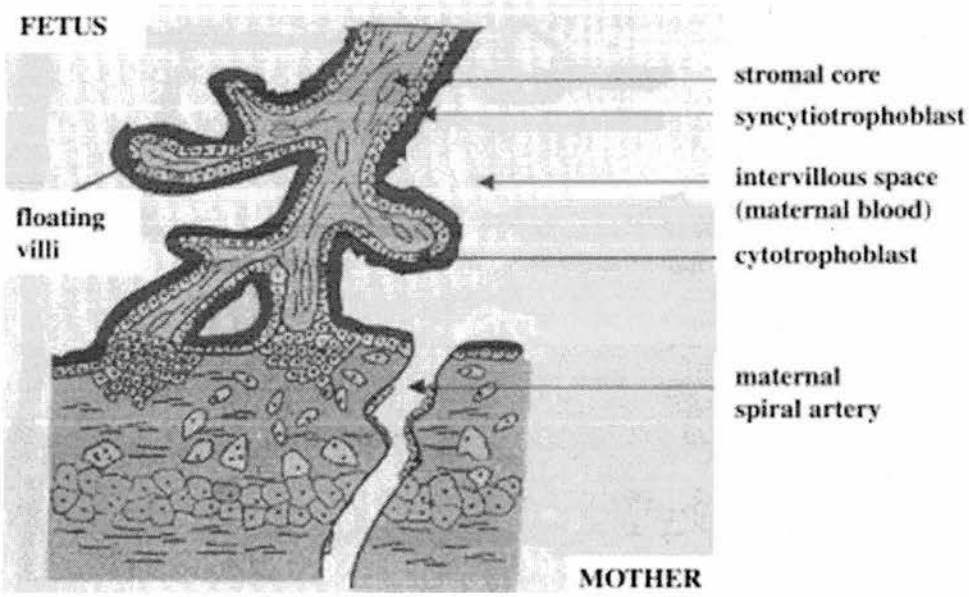


Figure 1.3: Schematic representation of the human materno–placental interface at mid-gestation. Chorionic floating villi are bathed with maternal blood in intervillous space (Evain-Brion & Malassine 2003).

1.2.1.5 Myometrium

The myometrium is the outer muscular portion of the uterus. It is composed of smooth muscle fibres which are made up of actin, myosin and intermediate filaments which are embedded in collagenous extra-cellular matrix. Myosin consists of 2 heavy (200 kDa) and 2 light chains (20 and 17 kDa) which are arranged functionally into the head and tail. The head contains the actin binding complex and has ATPase activity which is involved in contraction and relaxation. The tail plays a role in formation of the myosin filaments and transmission of muscle tension. Actin monomers (45 kDa) are polymerised into long, thin filaments which form cross-bridges with the myosin-heads to generate the contractile forces during uterine contractions. Intermediate filaments act to

link proteinaceous dense bodies with individual muscle fibres, helping to coordinate uterine contractions. Transmission and integration of myometrial contractility is further facilitated by connective tissue and the arrangement of fibres into random bundles (Huszar & Roberts 1982). Ion channels within the cell membrane can also act to regulate excitability of this muscle. The contractions of the myometrium will be discussed in section 1.2.2.3.

1.2.1.6 Cervix

The cervix has a high content of connective tissue, which is derived from collagen fibre bundles (Uldbjerg et al 1983a) embedded in a proteoglycan matrix (Kokenyesi & Woessner 1991). Collagens type I and type III are the predominant collagens present, with small amounts of collagen type IV. Elastin (Leppert et al 1983) and smooth muscle cells are also present. This connective tissue helps to resist stretch which is important in maintaining integrity and retaining the fetus in the uterus. Ripening of the cervix is one of the key steps in the coordinated control of parturition, as will be discussed in section 1.2.2.2.

1.2.2 Mechanisms of Parturition

Several parallel mechanisms work simultaneously during labour to result in delivery of the fetus. These include communication from the fetus – the maturation of the fetal pituitary-adrenal axis and the placental clock. Structural changes also occur, such as rupture of the fetal membranes and detachment of the placenta, and cervical ripening. Also, changes in excitability cause the myometrium to contract. Factors involved in the process of parturition will be discussed in this section, and are summarised in *Figure 1.4.*

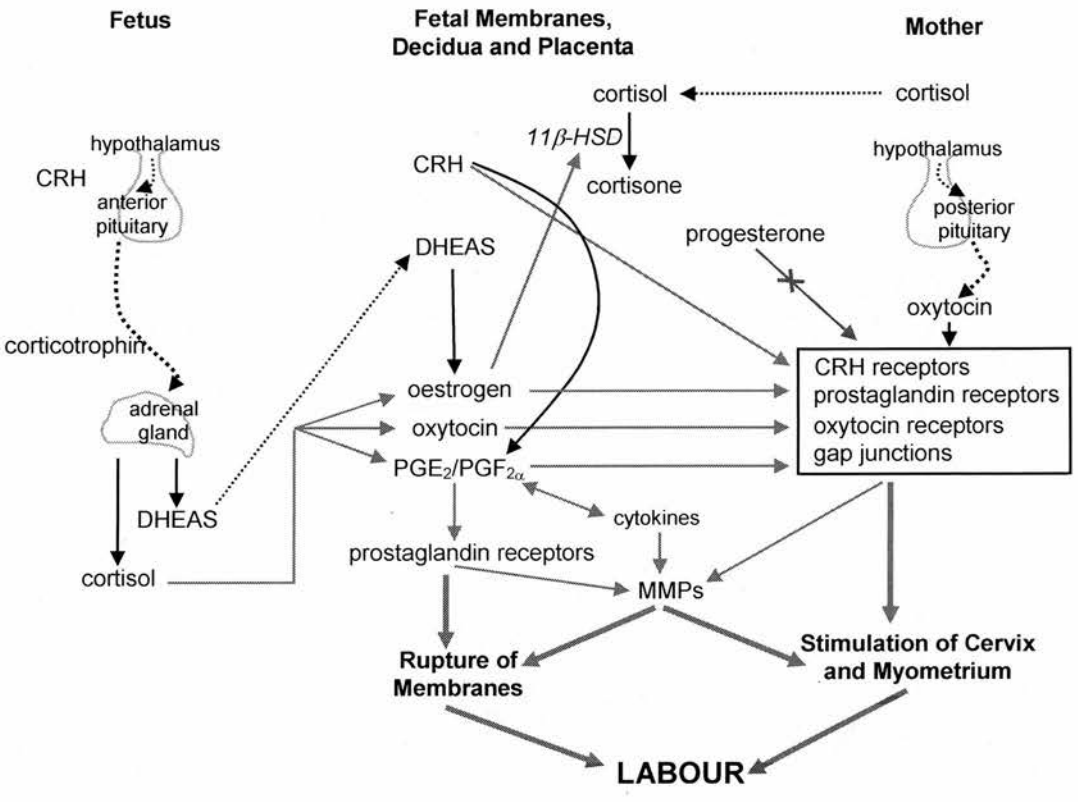


Figure 1.4: Diagram of the proposed mechanisms that have been implicated in the onset of labour, showing an integrated cascade of autocrine and paracrine factors (adapted from Norwitz et al 1999).

1.2.2.1 Tissue Remodelling and Rupture of the Membranes

In most pregnancies, labour begins at term in the presence of intact fetal membranes (Alger & Pupkin 1986). They usually remain intact until rupturing near the end of the first stage of labour. Rupture of the fetal membranes during labour is though to be caused by general weakening due to uterine contractions and repeated stretching. Experiments show that the tensile strength of the membranes is lower in labouring tissues, than those obtained from non-labouring caesarean section (Lavery et al 1982). The fetal membranes have been examined, and an area with unique morphological features identified along the line of rupture. This has been termed the 'zone of altered

morphology' (ZAM) and its features are consistent with structural weakness (Malak & Bell 1994b). These features consist of disruption of connective tissue layers and marked reduction in thickness and cellularity of the trophoblast and decidual layers. The zone does not include the entire rupture site, thus it may appear before membrane rupture. It also seems that the fetal membranes overlying the cervix are located at one end of the post-delivery tear. Due to these structural features, and its localisation to an area within the rupture line, it has been proposed that the ZAM likely arises from the cervical area of the fetal membranes and may have represented a site of initial weakness in response to the increased intra-amniotic pressures during labour (McLaren et al 1999).

Tissue remodelling of the fetal membranes plays a key role in their rupture. Changes involve decreased collagen content, altered collagen structure, and increased collagenolytic activity. The matrix metalloproteinases (MMPs - see section 1.5) are a family of enzymes which are capable of degrading virtually every component of ECM (Hulboy et al 1997). Degradation of collagen within the extracellular matrix of the fetal membranes is mediated primarily by MMPs. The integrity of the fetal membranes remains unaltered throughout most of gestation, partly due to low concentrations of MMPs and a higher concentration of their endogenous inhibitors, TIMPs (Vadillo-Ortega et al 1996). Near term, the MMP to TIMP activity balance switches in favour of degradation of the extracellular matrix. In human amnion, MMP-9 activity increases, and TIMP-1 concentrations decrease with the onset of labour (Vadillo-Ortega et al 1995, Vadillo-Ortega et al 1996) and in amniotic fluid, all TIMP levels decrease with labour (Riley et al 1999b). Other studies have shown that, in the fetal membranes, MMP-1 activity increases before labour, MMP-9 and MMP-3 activities increase during labour, and TIMP-1 concentrations increase after delivery, thus showing a pattern of controlled collagen degradation (Bryant-Greenwood & Yamamoto 1995).

Fetal membranes may rupture before the onset of labour. Premature rupture of the fetal membranes (PROM) defines rupture at term before labour begins, and preterm PROM (PPROM) defines membrane rupture before the onset of labour occurring preterm.

PPROM is one of the most common causes of preterm birth and will be discussed in section 1.2.3.

1.2.2.2 Cervical Ripening and Dilation

In order for the fetus to move from the uterus into the outside world, the nature of the pregnancy cervix must change by softening and dilating, permitting the fetal head to engage at the internal os and descend towards the end of pregnancy. This process is termed 'ripening'. The process by which this occurs involves two changes in the intercellular matrix: a reduction in collagen fibres and a marked increase in glycosaminoglycans (GAGs), which decrease the aggregation of the remaining collagen fibres. In the human cervix, keratin sulphate, which does not bind collagen, increases peripartum, and dermatan sulphate, which binds tightly to collagen, decreases. This aids the loosening of collagen bundles.

The profound collagen degradation occurring during cervical ripening is likely to be mediated by collagenases. Studies show that levels significantly increase within the cervix during the latter stages of cervical ripening (Osmers et al 1992, Rajabi et al 1988, Rechberger & Woessner 1993) and labour. MMP-8 and MMP-1, the two principal collagenases (see section 1.6) have distinct collagen specificities and are secreted by different cell types. MMP-8 is released in granules from neutrophils and cleaves collagen type I twenty times faster than collagen type III. MMP-1 is produced in fibroblasts and degrades collagen type III fifteen times faster than type I (Hasty et al 1987). Studies have shown a release of neutrophil derived MMP-8 (Hasty et al 1990) and an increase in leukocyte elastase activity (Uldbjerg et al 1983b) during cervical ripening. In-situ hybridisation studies have failed to show an increase in MMP-1 release by cervical fibroblasts during cervical ripening (Osmers et al 1992, Rath et al 1991). However, culturing of cervical fibroblast cells with PGF2a or IL-1a, or stimulation of these cells with cyclic mechanical stretch increase MMP-1 secretion, suggesting a role for MMP-1 in cervical ripening (Yoshida et al 2002).

Prostaglandins play a role in cervical ripening, in fact PGE_2 has been used for over 30 years to ripen the cervix and is still the most widely used and effective cervical ripening agent in current clinical practice (Calder & Embrey 1973, Kelly et al 2003). The mechanisms by which prostaglandins effect cervical remodelling are currently poorly understood. It is not clear whether reduction in cervical collagen caused by PGE_2 is a direct consequence of collagen degradation caused by an increase in collagenase activity (Rath et al 1987) or that prostaglandins may be modifying the composition of GAGs, altering the interaction of collagen with ECM (Calder & Greer 1991). PGE_2 may also act as a vasodilator and synergise with chemotactic agents to facilitate leukocyte infiltration into the cervix thereby effecting cervical ripening (Kelly et al 1994).

Cytokines have also been implicated in cervical ripening. Studies have demonstrated a significant increase in IL-8 concentration within the cervix (Kelly et al 1992, Sennstrom et al 1997) and lower uterine segment (Osmers et al 1995b, Winkler et al 1998) with levels correlating with MMP-8 and MMP-9 concentrations (Osmers et al 1995a). Other cytokines such as IL-1β and TNF-α have also been shown to induce cervical ripening in rabbits (El Maradny et al 1996) and guinea pigs (Chwalisz et al 1994), however the remodelling which they cause less closely resembles the physiological process.

Nitric oxide has also been proposed to play a role in cervical ripening (Chwalisz & Garfield 1998). There is down regulation of cervical iNOS expression during pregnancy and up-regulation at parturition in the rat (Ali et al 1997) and in guinea pigs, intra-cervical application of sodium nitroprusside, a nitric oxide donor, induces cervical ripening which is morphologically identical to the physiological process (Chwalisz et al 1997). In humans, the role of nitric oxide in cervical ripening is less clear. It has been demonstrated to induce clinically effective cervical softening in primiparous women compared to parous women, prior to pregnancy termination (Thomson et al 1997). However, there is evidence to suggest it is successful in arresting preterm labour with premature cervical dilation (Lees et al 1994, Rowlands et al 1996). However, this effect may be due to the tocolytic properties of nitric oxide and not the consequence of a direct

cervical effect. It is a possibility that, because nitric oxide interacts with prostaglandins, MMPs and cytokines, in addition to acting as a vasodilator, it is involved in pro-inflammatory remodelling cascades.

1.2.2.3 Myometrial Contractions

In the myometrium, the actin-myosin interaction is regulated by phosphorylation (contraction) or dephosphorylation (relaxation) of myosin light chains, and this occurs via myosin light chain kinase (MLCK) (Adelstein & Eisenberg 1980). The activity of this enzyme is tightly regulated. It is activated by binding of calmodulin and calcium and goes on to phosphorylate light chains of myosin. Phosphorylated myosin interacts with actin, forming a functional structural complex capable of converting the chemical energy of ATP into the mechanical energy of contractions. Inhibition of MLCK occurs via PKA. This phosphorylates MLCK, decreasing its affinity for myosin light chains, and it generates cAMP which facilitates calcium uptake into the sarcoplasmic reticulum, thus reducing free calcium.

In pregnant sheep and non-human primates, a pattern of myometrial activity known as contractures has been described (Nathanielsz et al 1995, Nathanielsz et al 1997). These are an intrinsic property of uterine muscle (Lye & Freitag 1988) and consist of prolonged, low amplitude increases in intrauterine pressure. Prior to parturition, contractures switch to uterine contractions, which are shorter, more regular and generate a higher intrauterine pressure. Studies show that this switch generally occurs at night and is related to alterations in maternal plasma oestrogen concentrations, and maternal oxytocin function (Nathanielsz et al 1995). Pregnant primate oxytocin levels have been shown to have a 24 hours rhythm, with the greatest levels apparent during the early hours of darkness (Honnebier et al 1989). These authors also showed that a competitive oxytocin antagonist inhibits the contractions. This increase in oxytocin may be due to oestrogen which has been shown to regulate oxytocin production as discussed in section 1.2.2.6.

Human myometrium can generate uncoordinated contractions, termed Braxton-Hicks contractions (Braxton-Hicks 1872) from early pregnancy. At parturition, the myometrium functions as a syncytium, facilitated by the expression of gap junctions within the myometrium, which increase in size and number (Ciray et al 1995) with the onset of labour. The concentration of gap junctions correlates with the frequency of uterine contractions and the stage of cervical dilation (Garfield & Hayashi 1981). It is suggested that the activation of the myometrium results from the coordinated expression of a cassette of contraction-associated proteins. Connexin-43, the myometrial gap junction protein, is one of such proteins, and the molecular mechanisms which control the expression of this gene have been analysed. Oestrogen is though to increase transcription of the gene. Since oestrogen plays a role in modulating myometrial contractility, this up-regulation of connexion-43 may be critical to the processes leading to increased synthesis of gap junctions at term, and hence contributing to the onset of labour (Lefebvre et al 1995).

1.2.2.4 Cytokines as Pro/Anti-Inflammatory Mediators

Cytokines are soluble protein or glycoprotein messengers that are released by immune cells and effect immune cell differentiation and function, in addition to other sites of synthesis and actions. They can regulate processes occurring in the extracellular environment by autocrine and paracrine means. They are relatively selective, binding to specific cell surface receptors, which are coupled to second messenger signalling systems and intracellular pathways. The cytokine family is large and diverse consisting of interferons, interleukins, growth factors and chemokines among others.

Helper T cells can be differentiated into 2 subgroups – Th1 and Th2. The cytokine environment at the time of Th0 cell stimulation determines the differentiation process. Generally, Th1 cells produce pro-inflammatory cytokines, and Th2 cells produce anti-inflammatory cytokines, though there is some overlap of cytokine classification, depending on the biological situation. At implantation, the cytokine balance at the feto-maternal interface is largely neutral, while on placentation it becomes biased towards the

Th2 type. By the third trimester of pregnancy, the general balance favours Th1-type cytokines, which continues on into parturition (Dealtry et al 2000, Lin et al 1993). In this section, the changes in expression of various cytokines with labour are discussed.

1.2.2.4.1 Interferons

Levels of IFN-γ produced by decidua, *in vitro*, were significantly lower in cells collected after labour compared to those before labour (Jones et al 1997). Contradictory studies report higher decidual and trophoblast IFN-γ mRNA at non-labouring term elective caesarean section compared to spontaneous vaginal delivery (Vives et al 1999). Also, it was found that IFN-γ levels increased in supernatants of placental, amnion and chorio-decidual homogenates following labour (Veith & Rice 1999). IFN-γ concentrations in amniotic fluid were found not to vary with labour (Olah et al 1996).

1.2.2.4.2 Pro-Inflammatory Cytokines

Patterns of expression of cytokines in the fetal membranes and decidua suggest that inflammatory activation occurs with term labour (Bowen et al 2002), but much more profoundly with preterm labour, particularly in the presence of intrauterine infection which promotes the process. The onset of labour at term induces elevations in amniotic fluid concentrations of IL-1β (Gunn et al 1996, Opsjln et al 1993, Romero et al 1990b), IL-6 (Gunn et al 1996, Laham et al 1996, Romero et al 1990a) and TNF-α (Opsjln et al 1993, Romero et al 1992b). Concentrations of these cytokines seem to vary in an interrelated way (Gunn et al 1996, Halgunset et al 1994) and to correlate with the amount of granulocyte filtration into the placenta (Halgunset et al 1994). Concentrations of inflammatory cytokines in cervicovaginal secretions increase during spontaneous term labour (Cox et al 1993, Steinborn et al 1996), peaking when the cervix is fully dilated. Cervicovaginal fluid levels of TNF-α increase profoundly after rupture of the fetal membranes (Steinborn et al 1996). Osman et al (Osman et al 2003) found that parturition was associated with significant mRNA increases of IL-1β, and IL-6 in the cervix and myometrium, IL-6 in the chorio-decidua and IL-1β in human amnion.

1.2.2.4.3 Anti-inflammatory Cytokines

In addition to pro-inflammatory cytokines, many gestational tissues also produce anti-inflammatory cytokines, such as IL-10 (Denison et al 1998). A decrease in levels of anti-inflammatory cytokines may lead to increases in pro-inflammatory cytokine levels which are associated with parturition. It has been reported that basal chorio-decidual production rates of IL-10 are significantly decreased with labour (Simpson et al 1998). No labour associated changes in IL-10 have been identified in amniotic-fluid (Dudley et al 1997, Greig et al 1995, Jones et al 1997) or in decidua (Jones et al 1997). IL-4 levels during term labour were only rarely decreased in amniotic fluid (Dudley et al 1996). TGF-β is present in amniotic fluid and is thought to maintain pregnancy by opposing the IL-1 and TNF-α action to stimulate prostaglandin production in intrauterine tissues (Bry & Hallman 1992, Bry & Hallman 1993) and also may act as a natural progesterone antagonist. MIC-1, a member of the TGF-β superfamily is also present in amniotic fluid, fetal membranes and decidua at term but changes in concentrations with labour have not been found (Keelan et al 2003).

1.2.2.4.4 Chemokines

Concentrations of IL-8 in amniotic fluid increase throughout pregnancy, and also with the onset of labour at term (Laham et al 1993, Romero et al 1991, Saito et al 1993). Levels of IL-8 in the lower segment of the uterus are also elevated with labour (Osmers et al 1995b). Some studies have indicated that IL-8 levels do not increase in explants from amnion, chorio-decidua and placenta during labour (Laham et al 1999), however other studies are contradictory to this. IL-8 production from placenta was reported to increase during labour (Elliott et al 1998) and from amnion and chorio-decidua (Keelan et al 1999a, Osman et al 2003). Concentrations of RANTES in amniotic fluid increased with the onset of term labour (Athayde et al 1999), as does GRO-α (Cohen et al 1996) though no changes were found in IL-16 associated with labour (Athayde et al 2000).

1.2.2.4.5 Other Cytokines

Leukaemia inhibitory factor (LIF) is undetectable in amniotic fluid during and at term of normal pregnancies, but can be detected after the onset of labour (Waring et al 1994). Uterine expression of LIF and its receptors suggests that it is involved in the establishment of pregnancy and reduced endometrial LIF contributes to human infertility (Cheng et al 2002, Vogiagis & Salamonsen 1999). Mice which lack the gene for the LIF receptor show abnormal growth and development of the placenta (Sharkey et al 1999). IL-15 mRNA and protein levels in the placenta increase following the onset of labour (Agarwal et al 2001).

1.2.2.5 Progesterone and Oestrogen

High progesterone levels in the uterine tissue are essential for initiation and maintenance of pregnancy. Progesterone acts locally within the uterus throughout pregnancy to suppress uterine contractions (Beck et al 1982) and decrease gap junction formation (Ambrus & Rao 1994). Almost 50 years ago, it was postulated that the removal of this progesterone block initiates labour and delivery (Csapo 1956). To date, evidence suggests that levels of progesterone are unaltered during labour. In the human, maternal, fetal and amniotic fluid progesterone levels do not decrease before the onset of labour (Boroditsky et al 1978, Walsh et al 1984). Progesterone is involved in the control of parturition though, as addition of synthetic progesterone antagonists (e.g. RU486) or addition of inhibitors of progesterone production (eg. inhibitors of the 3β-hydroxysteroid dehydrogenase enzyme such as epostane) initiate myometrial contraction and delivery (Selinger et al 1987). Thus, the role of progesterone could be carried out without need for alteration of the synthesis of this steroid. Methods involved in this progesterone control include sequestration of free active progesterone, inactivation of progesterone, production of a natural progesterone antagonist (eg. TGFβ), modulation of progesterone receptor coactivator and corepressor levels and changes in the levels of progesterone receptor subtypes (Mesiano 2004). NF-κB is constitutively active in human amnion at the time of labour and has been shown to repress progesterone receptor activity, thus

contributing to 'functional progesterone withdrawal' at the time of labour (Allport et al 2001).

Oestrogen plays a critical role in parturition by augmenting uterine contractility and excitability and increasing uterotonin production in the fetal membranes (Mesiano 2001, Pepe & Albrecht 1995). In human pregnancy, maternal oestrogens (oestrone [E1], oestradiol [E2] and oestriol [E3]) increase dramatically at mid-gestation. In the latter stages of pregnancy, concentrations of these hormones in the placenta are at levels 10 to 1000 times higher than in non-pregnant humans (Siiteri & MacDonald 1966). However, these changes in oestrogen levels do not appear to affect the fetal membranes and uterus. The oestrogens seem to exert their effects by increasing their bioactivity by interconversion of E1 and the more functionally active E2 (Madsen et al 2004b) by 17βHSD enzymes. This intracrine (see section 1.51) regulation controls the amount of biologically active E2 available to interact with the oestrogen receptors (ER) (Labrie et al 2000a, Labrie et al 2000b). The capacity to convert E1 to E2 (reductive activity) is high in human placenta and low in amnion, chorion and decidua, though enzyme activity is not affected by labour status in the placenta, amnion and myometrium, but only in the decidua and chorion (Madsen et al 2004b). The oxidative reaction (E2 to E1) predominates before labour, whereas after the onset of labour, the reductive conversion of E1 to E2 is greater (Mitchell & Wong 1993). Expression of ERα is increased in labouring tissue, whilst ERβ expression remains low and unaffected by the labour status (Smith et al 2002).

1.2.2.6 Oxytocin and the Oxytocin Receptor

Oxytocin is a small peptide hormone consisting of only nine amino acids, and was first identified through its potent and specific stimulatory activity for myometrial smooth muscle (Dale 1906). It is widely expressed in human tissue, including gestational tissue. Its receptor, OTR is a member of the vasopressin receptor family of G-protein coupled receptors. This receptor is expressed in human myometrium (Soloff et al 1979), decidua (Fuchs et al 1982), chorion and amnion (Chibbar et al 1993) and has not been detected in

the placenta (Wathes et al 1999). It is coupled to the $G_{\alpha q}$ (Ku et al 1995) which stimulates IP_3 production and intra-cellular Ca^{2+} release. This can stimulate the contractions which occur in the myometrium.

It has been shown that expression of OTR in the human myometrium increases in late pregnancy, with decidual and fetal membrane expression staying the same (Wathes et al 1999) and uterine synthesis of oxytocin increases after the onset of labour (Chibbar et al 1993). These findings suggest a paracrine action of oxytocin in intrauterine tissues to regulate myometrial contractions at labour.

It has been shown that IL-6 up-regulates OTR in cultured uterine smooth muscle cells (Rauk et al 2001) though contradictory results have shown IL-6 and IL-1β down–regulating myometrial OTR mRNA production (Schmid et al 2001). It may be expected that pro-inflammatory cytokines such as these would induce OTR expression alongside their role in promoting labour. Oestrogen seems to be a regulator of oxytocin synthesis. High plasma concentrations of oestrogen correlate with high oxytocin concentrations (Amico et al 1995) and treating decidual explants with oestrogen causes an increase in oxytocin mRNA and protein (Chibbar et al 1995). Oestrogen also stimulates OTR synthesis (Adachi & Oku 1995, Soloff 1975). Administering an oestrogen receptor antagonist in late pregnancy in the rat reduces oxytocin levels and delays parturition (Fang et al 1996). Progesterone also increases uterine oxytocin content (Fang et al 1997) though it reduces uterine OTR synthesis (Soloff et al 1983).

1.2.2.7 Fetal-Hypothalamus-Pituitary-Adrenal Axis

The concentration of cortisol in amniotic fluid increases during pregnancy in humans. The majority of cortisol present in the amniotic fluid at term is produced by the fetal adrenal (Schwartz & McMillen 2001), though the fetal membranes also produce some. Thus, the fetus is also involved in the onset of parturition. A number of theories as to how the fetal adrenal interacts with the placenta have been proposed.

It has been shown that in primary cultures of human placenta, cortisol is able to compete with the action of progesterone in the regulation of the corticotrophin-releasing hormone (CRH) gene by binding to the glucocorticoid receptor, thus preventing progesterone binding (Karalis et al 1996). CRH is a peptide highly expressed in human placenta at the end of gestation, which has been suggested to be involved in regulating the timing of parturition. These findings provide a model for functional progesterone withdrawal at the end of human pregnancy, which may be involved in the initiation of labour. CRH has also been shown to stimulate prostaglandin output via COX, and cytokine production from fetal membranes and decidua (Alvi et al 1999a, Alvi et al 1999b, Jones & Challis 1989). In fact the prelabour increases in COX-2 expression in the fetal membranes parallels that of CRH. These observations suggest paracrine/autocrine interactions between CRH and prostaglandins and cytokines and implicating CRH further as a placental signal for parturition.

Another hypothesis suggests that increased levels of oestriol at term stimulates placental 11-β-hydroxysteroid dehydrogenase (11-β-HSD), which converts cortisol to its inactive form, cortisone (Wilson & Parsons 1996). This lowers the exposure of the fetus to maternal cortisol. Negative feedback to the fetal pituitary releases corticotrophin, which stimulates fetal adrenal dehydroepiandrosterone sulphate (DHEAS) synthesis. DHEAS is then converted to oestriol in the placenta after being 16 α-hydroxylated in the fetal liver. The rise in placental oestriol production could then stimulate the release of oxytocin, prostaglandins and gap junctions, leading to myometrial contractions and cervical dilation.

1.2.2.8 Placental Clock

After isolation and characterisation of corticotrophin releasing hormone (CRH) (Vale et al 1981), it was discovered that this hormone was present in human plasma during the third trimester of pregnancy (Sasaki et al 1984). The CRH gene was then found to be expressed and synthesised in human placenta and fetal membranes (Grino et al 1987, Riley & Challis 1991, Warren & Silverman 1995). It is secreted into the maternal

circulation during gestation, where levels have been found to increase exponentially as pregnancy progresses (Campbell et al 1987, Goland et al 1986). The discovery of a high-affinity binding protein for CRH (CRH-BP) (Orth & Mount 1987) led to the finding that levels of this fell during the third trimester of pregnancy, leading to increased free CRH levels. It was then shown that women delivering prematurely had an early rise in CRH, and those delivering post term had a later rise than those delivering at term (McLean et al 1995). This led to the term 'placental clock' and it was postulated that the increase in CRH acted to control the length of human gestation. In support of this hypothesis, levels of CRH-type receptor 1 (CRH-R1) increase within the lower uterine segment with the onset of labour (Stevens et al 1998). In fact the myometrium expresses 4 different subtypes of CRH-R1 at term (Grammatopoulos et al 1998, Grammatopoulos et al 1999), indicating a role for these subtypes in parturition. The precise roles of CRH during pregnancy and parturition are unclear, however it is known that a series of molecular events mediated by autocrine, paracrine and endocrine actions of CRH are activated during pregnancy and at term to prepare the fetus and uterus for parturition (Hillhouse & Grammatopoulos 2002). The rise in CRH may contribute to relaxation of the lower uterine segment via CRH-R1 and to stimulation of myometrial activity either directly by binding to CRH-R2 and inducing calcium mobilisation, or indirectly by stimulating prostaglandin production (Jones & Challis 1989, Stevens et al 1998). Measuring plasma CRH during second trimester has been shown to potentially be a useful tool for predicting preterm labour (Inder et al 2001).

1.2.2.9 Lung Maturation and PAF in Parturition

The fetal lungs must be matured to ensure survival of the fetus outside the uterus. It follows therefore that the fetal lungs are directly involved in part of the initiation process of parturition. Platelet activating factor (PAF) was first identified as playing a role in this signal when it was found that PAF concentrations are higher in amniotic fluid from labour than non-labour (Billah & Johnston 1983, Hoffman et al 1990, Nishihira et al 1984). The lack of PAF secretion from the amnion and the fact that PAF has a direct role in lung maturation led to the proposal that the source of PAF was actually the lung

(Billah & Johnston 1983). PAF receptors have been detected in type II pneumonocytes (Eguchi et al 1994), cells which line the alveoli in the respiratory part of the lungs. Prior to labour, these cells change from storing a large amount of glycogen, to being glycogen poor, lipid rich cells with lamellar bodies containing surfactant (Bourbon et al 1982). This shows that the lungs are prepared for secreting surfactant into alveoli to prevent alveolar collapse once the fetus is breathing in the extra-uterine environment (Frenkel et al 1996). PAF has a role in glycogenolysis, reducing fetal pulmonary and hepatic glycogen stores (Hoffman et al 1988). PAF has also been shown to have a role in initiating uterine contractions (Nishihira et al 1984, Tetta et al 1986), and also to stimulate the transcription of COX-2 and release of prostaglandins from the fetal membranes and decidua (Alvi et al 1999a). Fetal surfactant has also been shown to be involved in prostaglandin production from fetal membranes. The surfactant is secreted from the fetal lung into amniotic fluid where concentrations increase with gestation, and arachidonic acid is released from the lipids of the surfactant, thus providing the amnion with a precursor for prostaglandin production (Bernal & Phizackerley 2000, Lopez Bernal et al 1988).

1.2.3 Preterm Labour

Preterm labour, as defined by the onset of labour prior to 37 weeks gestation, remains a major problem in obstetrics, accounting for 70 % of perinatal morbidity and occurring in around 10 % of births (Hack & Fanaroff 1993, McCormick 1985), a value which has not changed in the last 20-30 years (Challis et al 2002, Savitz et al 1991). Causes of preterm birth in general fall into three categories: iatrogenic; where there is a demonstrable complication of pregnancy such as pre-eclampsia or fetal distress that requires obstetrical intervention; premature rupture of the fetal membranes with or without infection; and idiopathic preterm labour. Generally, 30-40 % of preterm birth is associated with an underlying infective process, and 40-50 % of preterm births are idiopathic (Challis et al 2000). Preterm premature rupture of the fetal membranes (PPROM) is defined as rupture of the fetal membranes before the onset of labour, prior

to 37 weeks gestation. This occurs in approximately 5-10 % of pregnancies and is the leading cause of premature labour accounting for around 40 % of these deliveries.

Risk factors for preterm delivery include previous low birth weight or preterm delivery, multiple second trimester abortions, multiple gestations, placental anomalies, cervical and uterine anomalies, gestational bleeding, *in vitro* fertilisation pregnancy, hydramnios, infection, cigarette smoking, low socio-economic class and black race (Creasy et al 1980, Mercer et al 1996, Norwitz et al 1999). Presently, there are no effective diagnostic indicators of preterm birth, and no effective treatments for this condition. However, there are several biochemical markers which could potentially be useful indications of infection and the risk of preterm labour. As mentioned previously, cytokines and prostaglandins are elevated during preterm labour. Cervicovaginal fetal fibronectin detection can be a good indicator of preterm labour and PPROM (Goepfert et al 2000, Ramsey & Andrews 2003). Other markers have also been tested: CRH in maternal blood (McGrath et al 2002), intercellular adhesion molecule (ICAM-1) in chorio-decidua (Marvin et al 1999), β-hCG in cervicovaginal secretion (Bernstein et al 1998), MMP-9 in amniotic fluid (Harirah et al 2002), glucose concentration in amniotic fluid (Gonzalez-Bosquet et al 1999), and salivary oestriol elevation (Ramsey & Andrews 2003).

Infection induces a maternal and fetal inflammatory response which can lead to production and release of pro-inflammatory cytokines, which induce prostaglandin production leading to cervical ripening and myometrial contractility. These cytokines and prostaglandins are elevated in preterm delivery. Infection plays a key role in PPROM – there is an increased chance of infection occurring when the interval between rupture and delivery is elongated. Bacterial infection within the uterus can occur between the maternal tissues and fetal membranes, within the fetal membranes (chorioamnionitis), within the placenta (villitis), within the amniotic fluid (amnionitis), or within the umbilical cord or the fetus (funistis). The bacteria can invade the uterus from the abdominal cavity through the fallopian tubes, needle contamination, through

placental blood, or passage through the cervix from the vagina (Goldenberg et al 2000). Generally, very early, as opposed to later preterm deliveries are associated with intrauterine infection. Treating infection in women who have intact membranes but symptoms of preterm labour with antibiotics does not usually delay delivery, reduce the risk of preterm delivery, or improve the neonatal outcome (Gibbs & Eschenbach 1997). For women who present with rupture of the membranes near term, promoting delivery is usual to prevent intrauterine infection. However, if the rupture occurs prematurely, treatment should be directed to conserving the pregnancy and reducing neonatal morbidity due to prematurity. Antibiotic treatment can increase time to delivery and improve the outcome of the birth (Kenyon et al 2004, Mercer 2003, Mercer et al 1997).

1.2.4 Animal Models for Investigating Parturition

It is difficult to find a useful animal model of human parturition, since mechanisms involved are so very different in other species. These differences make it hard to extrapolate any animal data to humans. Some of the differences and similarities in events at parturition between species are compared in *Table 1.1*. In mice, many other rodents and rabbits, the corpus luteum is essential for progesterone synthesis and the maintenance of pregnancy. In these species, luteolysis determines the timing for the onset of labour and is mediated via activation of the FP receptor in the corpus luteum by $PGF_{2\alpha}$ (Cook et al 2003, Sugimoto et al 1997). Luteolysis causes a fall in maternal progesterone levels, which is rapidly followed by uterine activity and labour. The luteolytic mechanism does not apply in the human because the corpus luteum is only important for the early stages of pregnancy, after which it regresses. Progesterone withdrawal does not occur prior to parturition in the human, and the placenta is the primary source of progesterone production, and oestrogen production alongside the fetal adrenal. The fetal hypothalamic-pituitary-adrenal-axis appears to have a supportive rather than direct role in the human at parturition. In sheep, and many other mammals, activation of the fetal hypothalamic-pituitary-adrenal-axis and increased glucocorticoid production precedes labour. In the human, an increase in glucocorticoid production does

not precede labour, however, placental CRH may contribute to the timing of gestation see section 1.2.2.7 and 1.2.2.8).

Species	Steroid synthesis site at term	Progesterone changes at term	Oestrogen changes at term	Glucocorticoid changes at term	Site of prostaglandin production	Prostaglandin effects
Human	Placenta	No decrease	Gradual Increase	Little change	Uterus/fetal membranes	Uterine/gestational tissues
Non-human primate	Placenta	No decrease	Gradual increase	Little change	Uterus/fetal membranes	Uterine/gestational tissues
Sheep	Placenta	Decrease	Gradual increase	Increase	Uterus/fetal membranes	Uterine/gestational tissues
Mouse	Corpus luteum	Decrease	Increase	Large decrease	Uterus/fetal membranes	Luteolysis

Table 1.1: Comparison of parturition events in different species. Adapted from Gross et al 2000.

The mouse has been widely used to study parturition, despite the obvious differences between events which occur during parturition in the mouse and human. There are similarities in the changes associated with increased myometrial contractility during labour, for example contractile PG receptors increasing in the myometrium. Mice are also advantageous for the ability to create knockout models, and modify the mouse genome through transgenics (Majzoub & Muglia 1996) to assess gene function. The FP receptor knockout mouse, for example, fails at parturition (Sugimoto et al 1997). Whether results from mouse models of parturition can be extrapolated to human data though remains a problem with these studies. The sheep is also commonly used as a model for parturition. Increases in prostaglandin levels precede labour, as they do in the human, and uses of prostaglandin inhibitors in this model have been shown to inhibit premature labour (Grigsby et al 2000). The sheep has also been used to investigate the role of MMPs and TIMPs in tissue remodelling in the placenta (Riley et al 2000). Non-human primates offer several advantages for the study of parturition. They show the

same patterns of progesterone, oestrogen and glucocorticoid changes prior to parturition, and the placenta is the site of this steroid synthesis in late gestation, as opposed to the corpus luteum in some species. A great deal of work has been carried out on the involvement of prostaglandins and their receptors during pregnancy and parturition in the baboon (Nathanielsz et al 2004, Smith et al 1998, Smith et al 2001a, Smith et al 2001b) (as will be discussed in Chapter 3). It is possible to carry out *in vivo* studies in the baboon, to repeatedly sample blood and amniotic fluid, and electromyographic leads can be places on the myometrium to record uterine contractility (Nathanielsz et al 2004). Besides non-human primates, the data obtained from studies of parturition in animal models may not be very relevant to humans, and care must be taken in applying any findings to the human.

1.3 Prostaglandins

1.3.1 Discovery

In the 1930s, Kurzrok and Lieb were the first to observe the actions of prostaglandins when they stimulated isolated human uterine strips with fresh human semen and observed changes in contractility (Kurzrok & Lieb 1930). Subsequent independent studies confirmed this (Goldblatt 1933, von Euler 1934) with reported contractions in smooth muscle induced by a substance extracted from human seminal fluid and sheep seminal vesicle glands (von Euler 1968). These agents were named "prostaglandins" since they were apparently produced in the prostate gland (von Euler 1936). In non-pregnant women, Eliasson demonstrated in 1959 that prostaglandins, like human semen, caused spontaneous contractions of isolated myometrial strips, yet PGs administered intra-vaginally stimulated uterine contractions at the time of ovulation (Eliasson & Posse 1960). Subsequent studies showed that human seminal PG is secreted primarily by seminal vesicles (Eliasson 1959) and not the prostate and further investigation showed a variety of tissues produced PGs. They were confirmed to be fatty acids due to their solubility in lipid solvents and their acidic characteristics. The chemical structures of naturally occurring PGs were determined (Bergstroem et al 1963) and subsequently, a

variety of PGs and related eicosanoids with profound biological activities have been isolated and characterised.

1.3.2 Nomenclature

All prostanoids exhibit roughly the same structure as they are all oxygenated fatty acids composed of 20 carbon atoms and containing a cyclic ring, a C-13 to C-14 *trans*-double bond and a hydroxyl group at C-15. Prostanoids can be classified into prostaglandins which contain a cyclopentane ring, and thromboxanes which contain a cyclo-hexane ring. The prostaglandins are classified into types A to J, according to modifications of the cyclo-pentane ring. A, B, C and J are thought not to occur naturally, but are metabolites of the other prostaglandins. Thus, the naturally occurring prostaglandins consist of prostaglandin D (PGD), PGE, PGF, and PGI. The thromboxanes are divided into TxA and TxB, TxB being the stable metabolite of TxA. The abbreviations are commonly followed by an index (for example PGE_2) which indicates the number of double bonds present in the various side chains attached to the cyclo-pentane ring.

1.3.3 Biosynthesis and Catabolism of Prostaglandins

The principal precursor of the prostanoids is the poly-unsaturated fatty acid, arachidonic acid (AA). AA is released from membrane phospholipids (*Figure 1.5*) by phospholipase A_2 (PLA_2) in response to various physiological and pathological stimuli. Around 10 distinct PLA_2 groups have been identified and can be categorised into secretory ($sPLA_2$), cytosolic Ca^{2+}-dependent ($cPLA_2$) and cytosolic Ca^{2+}-independent ($iPLA_2$) (Dennis 1997). AA is converted to the prostanoid precursor PGG_2, which is subsequently peroxidised to PGH_2. Both these reactions are catalysed by the protein cyclo-oxygenase (COX) which predominantly consists of 2 forms: the endoplasmic reticulum-localised COX-1 (Hla et al 1986, Needleman et al 1986), and COX-2 (Hla & Neilson 1992) which acts predominantly at the nuclear envelope (Morita et al 1995). COX-1 has been shown to be constitutively expressed, with COX-2 being the inducible form (Morita 2002). COX enzymes are also known as prostaglandin H-synthases (PGHS-1 and -2). A third

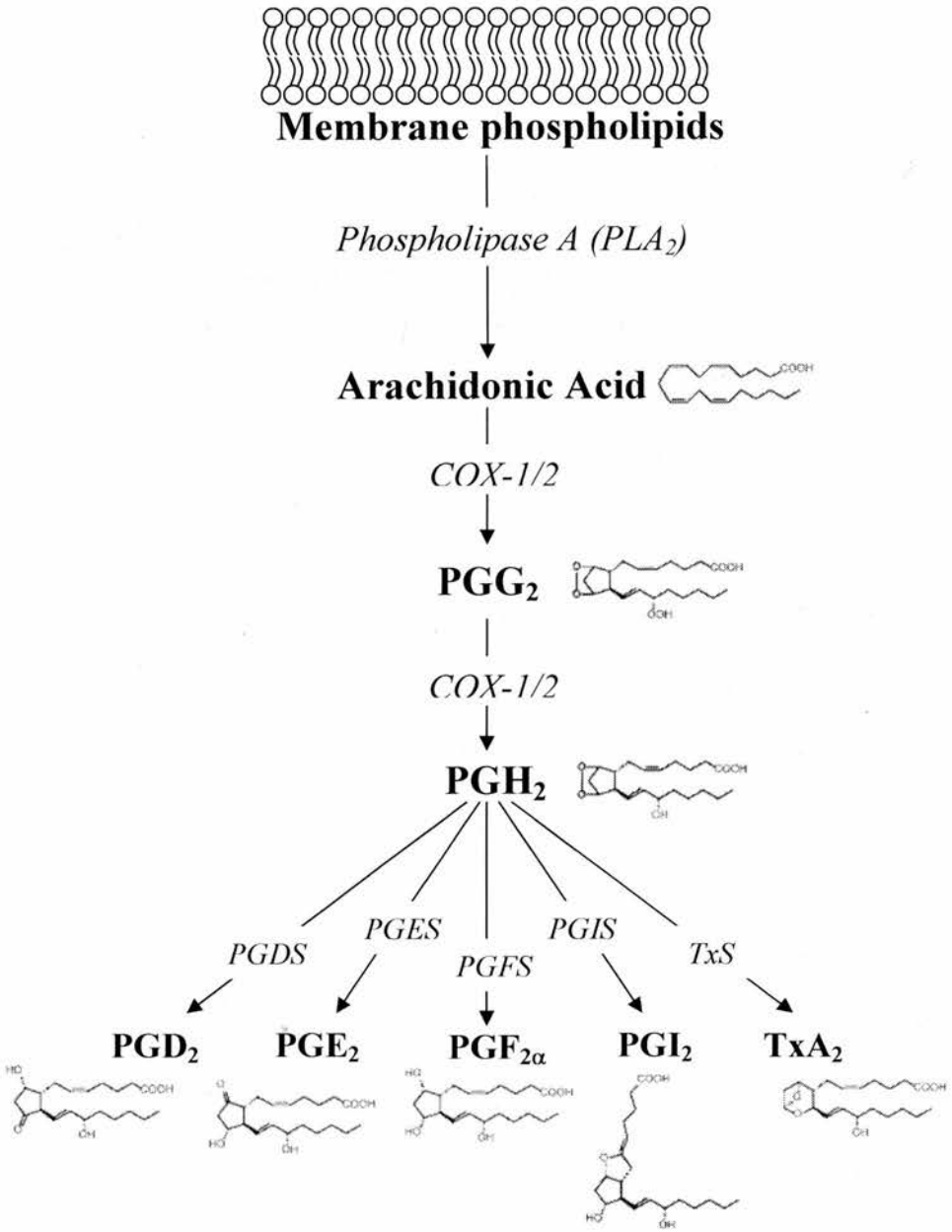


Figure 1.5: Prostaglandin biosynthesis. Arachidonic acid is liberated from membrane phospholipids by phosopholipase A_2 (PLA_2) and then is converted to the prostaglandin endoperoxidases, PGG_2 and PGH_2 via cyclo-oxygenases 1 and 2 (COX-1 and -2). Specific synthases then act to synthesise the specific prostaglandins. Prostaglandin structures reproduced from Hata & Breyer 2004.

form, termed COX-3, which is a splice-variant of COX-1 has recently been identified (Chandrasekharan et al 2002), though its role in human physiology and pathophysiology remains to be established. Following COX activity, prostanoid synthesis is completed by specific synthases, for example PGES for PGE_2. The important role of prostanoid biosynthesis in pathophysiology is demonstrated by the well-known clinical effects of pharmacological COX inhibitors. Non-steroidal anti- inflammatory drugs (NSAIDs) such as aspirin and indomethacin act by inhibiting COX enzymes (Mitchell et al 1993, Sadovsky et al 2000). It was originally thought that release of AA from membrane phospholipids was the rate limiting step in the synthesis of prostaglandins (Lands 1979), however, COX enzymes have also been implicated as the rate limiting step in this process (Needleman et al 1986, Smith & Marnett 1991).

Biologically active PGs are rapidly broken down either by hydrolysis or conversion to inactive metabolites. PGE_2 and $PGF_{2\alpha}$ are metabolised by the cytosolic enzyme, NAD^+ dependent 15-hydroxyprostaglandin dehydrogenase (PGDH) to form carbon-15 keto derivatives (Anggard & Samuelsson 1965). This is followed by a reduction of the Δ^{13} double bond, catalysed by Δ^{13}-15-ketoprostaglandin reductase, to the 13,14-dihydro-15-keto metabolites (PGEM, PGFM) (Anggard et al 1971, Anggard & Samuelsson 1964). These enzymes are ubiquitously expressed in mammalian tissues (Tai et al 2002) and the metabolites, PGEM and PGFM have significantly reduced biological activity (Anggard 1966). PGE_2 can also be metabolised by 9-ketoreductase to form $PGF_{2\alpha}$ and then hydrolysed to PGFM (Jarabak 1980).

Prostaglandins have a short half life and are metabolised rapidly, suggesting their actions are local to their production site. Newly synthesised prostaglandins diffuse across the plasma membrane into the extracellular compartment driven by pH and membrane potential. However, metabolic clearance requires energy dependent uptake across the plasma membrane followed by cytoplasmic oxidation. It has been suggested that carrier-mediated PG transport is involved in degradation. Several prostaglandin carriers have been cloned (Schuster 2002). Human prostaglandin transporter (hPGT) is a lactate/PG

exchanger and is expressed in COX containing cells (Lu et al 1996). Another prostaglandin transporter, organic anionic transporter (OATP) has lower affinity and specificity for PGs. PG transporters, besides being involved in metabolic clearance, are also involved in the re-uptake of PG in PG synthesising cells (Bao et al 2002). This reuptake may control the net release of PGs as a negative feedback loop when PG synthesis is stimulated, or in quiescent cells to maintain a basal level of PG. PG reuptake within the cell via PGT can also provide intracellular access to nuclear membrane located receptors as will be discussed in section 1.4.5.

1.3.4 Prostaglandin Synthesis and Catabolism During Pregnancy and Parturition

PG production is compartmentalised within pregnant maternal and fetal tissues (Challis et al 2000). Arachidonic acid is present in the phospholipid layer of fetal membranes (Schwarz et al 1975). The amount of arachidonic acid in the fetal membranes increases during late gestation (Curbelo et al 1981), and decreases after the onset of labour (Okita et al 1982, Schwarz et al 1975). Levels of free arachidonic acid found in the amniotic fluid increase after the onset of labour (Keirse et al 1977, MacDonald et al 1974). This suggests that arachidonic acid released from the amnion diffuses into the amniotic fluid.

Phospholipase A_2, which catalyses the release of the arachidonic acid, is also present in the human fetal membrane (Grieves & Liggins 1976, Schultz et al 1975). Activity was higher in the decidua and amnion than in the chorion and myometrium and levels and activity did not appear to be different in labouring tissues than in non-labouring tissues (Grieves & Liggins 1976). Recently it was found that labour is associated with increased expression of type-IIA $sPLA_2$ but not type IV $cPLA_2$ in human myometrium (Slater et al 2004).

Human amnion is a major site of PG synthesis, predominantly PGE_2 (Challis & Olson 1988, Gibb & Sun 1996), both COX-1 and COX-2 have been identified there (Rose et al 1990, Teixeira et al 1994). There are only very low PGDH levels in the amnion (Cheung et al 1990, Keirse & Turnbull 1975, Okazaki et al 1981). However, in the chorion there

are very high PGDH levels which have been localised to the trophoblast layer (Cheung et al 1990, van Meir et al 1997). COX is also abundant in this tissue (Gibb & Sun 1996). The decidua contains low concentrations of COX-1 and COX-2, and little PGDH (Cheung et al 1990, Okazaki et al 1981). Overall, in the amnion, chorion and decidua PG output, and COX-2 mRNA increase at the onset of labour (Gibb & Sun 1996, Skinner & Challis 1985, Slater et al 1998, Slater et al 1995). Evidence suggests that although PGDH levels do not change in the amnion and decidua (Skinner & Challis 1985), in the chorion, mRNA expression and activity of PGDH decrease in human labour, at term and preterm (van Meir et al 1997, Van Meir et al 1997, Van Meir et al 1996). Although amnion derived prostaglandins increase at labour, whether these can contribute to the prostaglandin stimulation of cervical ripening and dilation and myometrial contractility is uncertain. In fact, the amnion produces little of the PGE_2 detected on the decidual side of the fetal membranes (Sullivan et al 1993) most likely due to the high PGDH activity of the chorion (Cheung & Challis 1989, Sullivan et al 1992).

In the human myometrium, COX-1 and COX-2 levels are greater in the pregnant compared to the non-pregnant state (Moonen et al 1984), although there is controversy over changes of this enzyme level with the onset of labour. It has been reported to increase (Erkinheimo et al 2000), decrease (Zuo et al 1994) and stay the same (Giannoulias et al 2002, Moore et al 1999). A significant decrease in PGDH protein levels and activity is found in the myometrium with labour at term and preterm (Giannoulias et al 2002).

The amnion and chorio-decidua show no differences in amounts of either PGES at term or preterm, with or without labour, or advancing gestation (Meadows et al 2003), although a report suggests that chorion PGES activity does increase with labour (Alfaidy et al 2003) and decrease in the placenta. No labour associated changes in the expression of PGFS within human gestational and intrauterine tissues have been observed (Slater et al 2002). In the sheep, PGFS mRNA has been shown to increase following induced-

spontaneous labour in the placenta, but not in the myometrium. PGES mRNA levels did not change (Palliser et al 2004).

1.3.5 Role of Prostaglandins During Labour

Prostaglandins, particularly those produced within the intrauterine and gestational tissues, play many distinct roles during the initiation and progression of labour (Challis et al 2000, Novy & Liggins 1980). It is thought that PGs play a role in rupture of the fetal membranes through the stimulation of matrix metalloproteinase (MMP) activity subsequent to extracellular matrix protein remodelling. It has been reported that PGE_2 stimulates MMP-9 production in human fetal membranes (McLaren et al 2000b) and $PGF_{2\alpha}$ increases production of MMP-2 and -9 and an increase in activation of MMP-2 (Ulug et al 2001). It is widely accepted that PGs play a role in cervical ripening and dilation (Fletcher et al 1993, Keirse et al 1983). There is evidence that PGs play a role in placental separation (although this process is largely mechanical). A link between elevated human $PGF_{2\alpha}$ plasma levels and placental separation has been reported (Noort et al 1989) and also a link between administered PGE_2 at the induction of labour and placental abruption (Leung et al 1987). In cows with retained fetal membranes, due to the non-separation of the fetal and maternal cotyledons, placenta tissue concentrations of $PGF_{2\alpha}$ were significantly lower than in normally separated cotyledons at 6 hours after parturition (Takagi et al 2002). Uterine involution is very closely associated with plasma levels of $PGF_{2\alpha}$ in cows and sheep (Lindell & Kindahl 1983, Thompson et al 1987) and the administration of $PGF_{2\alpha}$ promotes uterine involution in cows (Lindell & Kindahl 1983).

There is a lot of evidence to suggest that PGs also play a role in myometrial contractility. It is well accepted that this involves enhanced uterine activation as well as increased levels of contractile stimulants. Several uterine activation proteins (UAPs) enhance the ability of the myometrium to respond to contractile agonists and to develop and propagate the depolarisation signal efficiently between smooth muscle cells (Olson 2003). These UAPs include the prostaglandin receptors, FP and EP1-4 and the

stimulators include PGs. PGs are now recognised as a trigger of labour because the myometrium contracts in response to exogenous PGs in vivo and in vitro (Crankshaw & Gaspar 1992, Garrioch 1978, Senior et al 1991, Senior et al 1993, Senior et al 1992, Wikland et al 1982, Wikland et al 1984, Word et al 1992). PG synthetic enzymes and levels in tissues and fluids increase before or at the time of labour (Hirst et al 1998, Hirst et al 1995, Mijovic et al 1999, Mijovic et al 1998, Slater et al 1995, Slater et al 1999), and inhibitors of PG synthesis delay birth and prolong pregnancy (Challis & Olson 1988, Poore et al 1999).

1.4 Prostaglandin Receptors

1.4.1 Identification of Prostaglandin receptors

Prostanoid receptors are a family of seven-transmembrane G-protein coupled receptors (GPCRs). These receptors have been cloned in humans and were first defined pharmacologically as EP, FP, DP, IP and TP, which referred to the endogenous ligands which activated these receptors: PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2 and TxA_2, respectively (Coleman et al 1994, Pierce et al 1995). There is a lot of heterogeneity of the prostanoid receptors due to the presence of genes encoding separate subtypes and also alternative mRNA splicing. There is however, more homology with each other than for example, with the histamine H1 receptor, suggesting a common ancestral prostaglandin receptor. Although each prostaglandin binds with the highest affinity to its cognate receptor, cross-reactivity can be observed between a given prostanoid and other receptors within the family (Breyer et al 2001). *Table 1.2* shows inhibition constants (Ki) for ligands interacting with prostanoid receptors.

Ligand	Ki (nM) EP1	EP2	EP3	EP4	FP	DP	IP	TP
PGE_2	9.1 ± 1.5	4.9 ± 0.5	0.33 ± 0.3	0.79 ± 0.07	119 ± 12	307 ± 106	>100000	29000 ± 6702
$PGF_{2\alpha}$	547 ± 104	964 ± 64	38 ± 6	288 ± 27	3.2 ± 0.3	861 ± 139	>100000	8700 ± 670
PGD_2	5820 ± 1801	2973 ± 100	421 ± 60	1483 ± 189	6.7 ± 0.5	1.7 ± 0.3	>100000	6602 ± 541
Iloprost	11 ± 1	1870 ± 176	56 ± 6	284 ± 9	619 ± 105	1035 ± 171	11 ± 1	6487 ± 29
U46619	28200 ± 3863	12020 ± 1617	13013 ± 2956	2013 ± 149	241 ± 8	3970 ± 390	53350 ± 3950	35 ± 5

Table 1.2: Binding and cross-reactivities between prostanoid receptors and their ligands. Prostacyclin and thromboxane, the endogenous ligands for IP and TP are unstable, so the most commonly used mimetics have been analysed in their place, iloprost for prostacyclin and U46619 for thromboxane. Data obtained from Abramovitz et al using human embryonic kidney cells (HEK293) expressing recombinant human prostanoid receptors (Abramovitz et al 2000).

1.4.2 EP Receptors

There are 4 subtypes of the EP receptor; EP1, EP2, EP3 and EP4, which are products of separate genes. These receptors use alternate, and in some cases opposing intracellular pathways (Ashby 1998) (*Figure 1.6*). PGE_2 interaction with EP1 mobilises intracellular calcium and inositol triphosphate (IP_3) via $G_{\alpha q}$. Activation of EP2 and EP4 result in stimulation of cAMP production via $G_{\alpha s}$ (Narumiya et al 1999, Sugimoto et al 1993). There are several splice variants of EP3 which are coupled to various signalling pathways resulting in either a positive or negative cAMP response to PGE_2 or an increase in intracellular calcium mobilisation and accumulation of IP_3. This depends on the splice variant and the cell type in which PGE_2 is acting. However, the nature of the G-protein activated by a specific prostaglandin receptor may sometimes differ between cell types. Although EP2 and EP4 have similarities in their functional coupling, comparing the primary sequences of these cloned receptors shows that they share only 38 % amino acid identity in their transmembrane domains. This is not dissimilar from the amino acid identity they share with EP1 (37 %) and EP3 (34 %). The cloned IP and

DP receptors actually have more sequence similarity with EP2 (60%) (Toh et al 1995). This suggests that second messenger coupling has more influence than ligand binding specificity with respect to the overall primary sequence of these receptors. It is surprising how dissimilar the signalling pathways for EP2 and EP4 are, given that EP2 is 358 amnio acids long, and EP4 is 488. EP4 also has 25 extra amnio acids in the third intracellular loop compared to EP2 (Regan 2003). The longer C-terminal end of EP4 consists of many serine and threonine residues which are potential phosphorylation sites. This C-terminal has been shown to be involved in short term agonist induced desensitisation in EP4 which is absent in EP2 (Bastepe & Ashby 1997, Nishigaki et al 1996). However, these serine and threonine residues have been shown to not play a role in internalisation (Desai et al 2000).

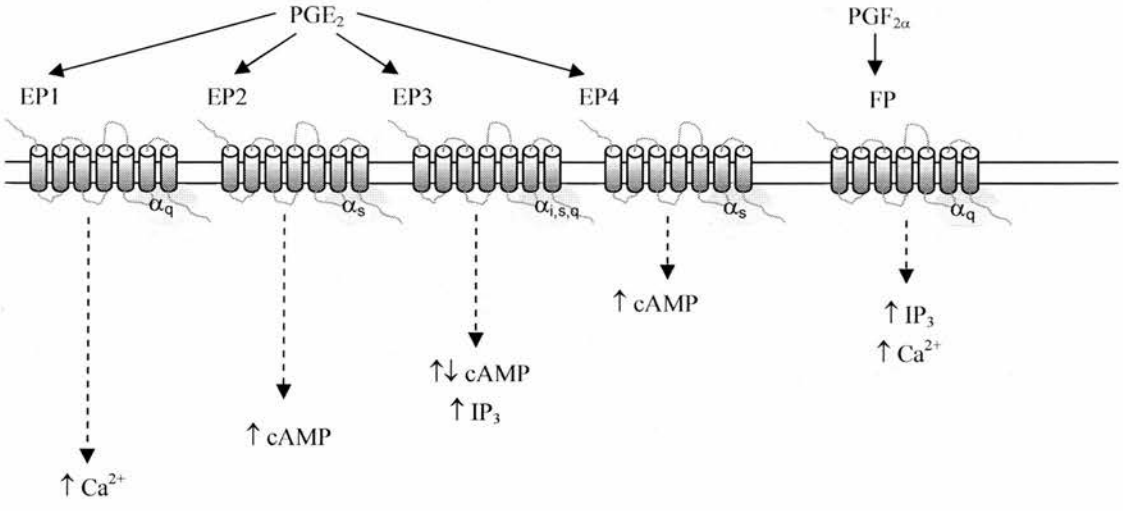


Figure 1.6: Prostaglandin receptors EP1-4 and FP structure and signalling. Adapted from Sales & Jabbour 2003.

1.4.3 FP Receptors

There are 2 known different forms of FP; FP_A and FP_B resulting from alternative mRNA splice variants. These splice variants are identical except for the carboxyl-terminal domain, which is truncated in the FP_B isoform and lacks the last 46 amino acids on the C-terminal domain (Fujino et al 2002). Both isoforms of FP are coupled to $G_{\alpha q}$ and can activate IP_3 via activation of phospholipase C, intracellular calcium flux and activation of protein kinase A. Also, both forms can activate Rho, one of the Ras-family of small GTPases, which in turn leads to phosphorylation and activation of focal adhesion kinase (FAK) associated with cell morphology (Pierce et al 1999).

1.4.4 Prostaglandin Receptors During Pregnancy and Parturition

EP1, 2, 3, 4, DP, FP and IP receptors have all been identified in the human pregnant myometrium (Erkinheimo et al 2000, Leonhardt et al 2003, Matsumoto et al 1997b, Myatt & Lye 2004, Senior et al 1993). It was found that EP3 and FP mRNA levels in the myometrium were down-regulated during pregnancy, possibly playing a role in the relaxation of the myometrium, thus aiding the maintenance of pregnancy (Matsumoto et al 1997b). EP2 levels can also be seen to decrease with advancing gestational age, implying that these too are involved in uterine quiescence (Leonhardt et al 2003). Brodt-Eppley et al investigated preterm and term, labouring and non-labouring lower segment human myometrial samples. They found EP2 mRNA levels were greater in preterm, no labour samples, and that FP mRNA levels were greater in term labour (Brodt-Eppley & Myatt 1999). These results are consistent with the theory that EP2 has a role in maintenance of uterine quiescence, whereas FP has contractility effects. There is not a lot of other published evidence of prostaglandin receptors in any other gestational tissue besides the myometrium in the human, though FP has been detected in human amnion-decidua complexes (Fukai et al 1984) and also in the human placenta (Vielhauer et al 2004).

Expression of prostaglandin receptors has been investigated in baboon chorion and decidua during parturition (Smith et al 2001b). It was shown that there was a lower

level of expression of the EP2 gene in decidua obtained from animals in spontaneous labour to those which were not in labour. This suggests that an adenylate cyclase-mediated effect may inhibit parturition-related processes in decidua in cells expressing the EP2 receptor. This parallels decreased EP2 receptor expression in myometrium and cervix (Smith et al 1998). In the chorion, a decrease in EP4 mRNA was also seen with advancing gestational age (Smith et al 2001b). This suggests an adenylate cyclase-mediated effect may inhibit changes in chorion which promote parturition. This is similar to the myometrium, where adenylate-cyclase coupled receptors inhibit myometrial contractility (Senior et al 1993).

EP receptor localisation has been investigated in the mouse uterus (Katsuyama et al 1997). Abundance and localisation of EP2 and EP4 mRNA changes considerably when mice undergo pseudo-pregnancy by treatment with pregnant mare's serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG). EP2 mRNA is barely detectable before stimulation and is expressed considerably in luminal epithelial cells on stimulation, peaking after around 5 days then disappearing.

It has been shown that mice lacking the FP receptor have failure of parturition (Sugimoto et al 1997). Although these mice showed no abnormality in the oestrous cycle, ovulation, fertilisation or implantation, they did not respond to exogenous oxytocin receptor (a proposed triggering event in parturition), and they did not show the normal decline of serum progesterone concentrations that precedes parturition. Ovariectomy restored induction of the oxytocin receptor and allowed successful delivery in the FP-deficient mice. This shows that parturition is initiated when $PGF_{2\alpha}$ interacts with FP in ovarian luteal cells of the pregnant mice to induce luteolysis, an essential process in this animal model.

1.4.5 Nuclear Prostaglandin Receptors

It has become clear recently that, as well as activating cell surface receptors, prostaglandins can activate nuclear receptors (Bhattacharya et al 1999, Bhattacharya et

al 1998). This gives rise to the possibility of many intracrine (see section 1.5.1) signalling pathways. As discussed previously, the PGT allows PG reuptake within the cell, and this can provide intracellular access to nuclear membrane located PG receptors. More evidence for the nuclear site of PG receptors comes from studies of the subcellular localisation and compartmentalisation of enzymes of the biosynthetic pathway, many of which have a perinuclear localisation (Gilmour & Mitchell 2001). The methods of action of the nuclear PG receptors are under investigation. In the brain, the activated G-protein coupled EP3 receptor can activate perinuclear Ca^{2+} dependent K^{+} channels, PI-3 kinase, MAPK and NF-κB pathways (Gobeil et al 2002). It is not yet clear whether nuclear PG receptors are present in gestational tissues (Helliwell et al 2004). Prostaglandins can also act on other nuclear receptors, namely the peroxisome proliferator activated receptors (PPARs). In fact the J series of prostaglandins is thought to not mediate its action via a G-protein coupled receptor, but via PPARs (Miwa et al 2004). These will be discussed in more detail in Chapter 3.

1.5 Signalling Pathways of the Prostaglandin Receptors

1.5.1 Endocrine, Paracrine, Autocrine and Intracrine Signalling

Signalling by extracellular secreted molecules can be subdivided into: endocrine, paracrine and autocrine (Snyder 1985). In endocrine signalling, a molecule acts on target cells different from their site of synthesis by cells of endocrine organs. Usually the molecule is carried in the blood from the release site to its target. Paracrine signalling involves the signalling molecules released by the cells only affecting target cells in close proximity. Autocrine signalling involves cells responding to substances that they themselves have released. Recently, a new term has been coined, namely 'intracrine' which describes active hormones which exert their action in the same cells where synthesis took place, without release into the pericellular compartment (Labrie 1991). Furthermore, molecules may act by more than one means of signalling.

1.5.2 Ligand-Receptor Binding

A signalling molecule acts as a ligand which binds to its site on its receptor inducing a conformational change in the receptor causing reactions leading to the specific cellular response (Alberts et al 1994). The function of the ligand is only to bind the receptor, it is not metabolised or an intermediate product in a reaction, and it does not have enzymatic properties. Target cells may modify or degrade the ligand, thus modifying or terminating its response, or the response of neighbouring cells to the signal.

Hormones can be classified on their solubility and receptor location (Kahn 1976). There are 3 categories, including small lipophilic molecules that diffuse across the plasma membrane and interact with intracellular receptors. There are also hydrophilic or lipophilic molecules that bind to cell surface receptors. Intracellular responses include those of steroid hormones, progesterone, cortisol, oestradiol and testosterone. Water soluble molecules acting via cell surface receptors include insulin and glucagons, and lipophilic molecules acting on cell surface receptors include eicosanoid hormones e.g. prostaglandins.

1.5.3 Cell Surface Receptors

These can be roughly organised into 4 categories. There are G-protein coupled receptors (GPCRs), receptor tyrosine kinases, tyrosine kinase linked receptors, and receptors linked to other enzymatic activities. GPCRs comprise the largest family of all cell surface receptors (see section 1.5.4). Receptor tyrosine kinases are the receptors for most growth factors and have intrinsic enzymatic activity. Ligand receptor interaction leads to receptor dimerization, which activates tyrosine kinase and phosphorylation of tyrosine on one receptor tail by another (transphosphorylation). Phosphotyrosine recruits effector molecules which are either enzymes or adaptors. Tyrosine kinase linked receptors lack intrinsic catalytic activity. Ligand binding stimulates formation of a dimeric receptor which interacts with and activates one or more cytosolic protein tyrosine kinase. Receptors linked to other enzymatic activities include protein tyrosine phosphatases,

protein serine/threonine kinases and guanylate cyclase. They are capable of autophosphorylating residues in their own cytosolic domain and phosphorylating various substrate proteins.

1.5.4 G-Protein Coupled Receptors

GPCRs are the largest family of cell surface receptors with over 100 members defined in mammals so far (Gether et al 2002, Lameh et al 1990). They mediate the cellular responses to a huge diversity of signalling molecules, including hormones and neurotransmitters which are highly variable in structure and function. The same ligand can activate many different family members. Despite this diversity, all of the GPCRs known to date have a similar structure, involving seven transmembrane segments, suggesting that they are evolutionarily related.

1.5.5 Trimeric GTP-Binding Proteins

Trimeric GTP-binding proteins (G-proteins) are GTPases and function as molecular switches that can flip between 2 states: active, when GTP is bound, and inactive, when GDP is bound (Bourne et al 1991, Hepler & Gilman 1992). When an extracellular signalling molecule binds to a GPCR, the receptor changes its conformation and switches on the trimeric G-proteins associated without causing them to exchange their bound GDP for GTP. The G-protein then dissociates into G_α and $G_{\beta\gamma}$ subunits and it is usually the G_α subunit that diffuses away, delivering its message downstream. The switch is reversed when the G-protein hydrolyses its own bound GTP back to GDP. Most GPCRs activate a chain of events altering the concentration of intracellular signalling molecules known as the second messengers.

1.5.6 Second Messengers

Binding of a ligand to many cell-surface receptors leads to short lived increases and decreases in the concentration of various second messengers e.g. cAMP, cGMP, DAG, IP_3 and Ca^{2+}. These are generated in large numbers and rapidly act within the cell to

broadcast the signal to other parts of the cell. This triggers rapid alteration in the activity of one of more enzymes or non-enzymatic proteins.

1.5.7 Cyclic AMP (cAMP) Pathway

cAMP can activate protein kinase A (PKA), which in turn can activate transcription of certain genes. Thus a high or low concentration of cAMP can switch on or off target genes. cAMP is a nucleotide that is generated from ATP by adenyl cyclase in response to stimulation of many types of cell-surface receptors. cAMP acts as an intracellular signalling molecule by activating cAMP-dependent kinase, protein kinase A (PKA) (Krebs 1989). Extracellular signals can cause changes of more than 20 fold in seconds. This requires that rapid synthesis of cAMP is balanced by its rapid breakdown or removal. It is hydrolysed to AMP by a phosphodiesterase. However, the extracellular signal causes the increase in cAMP activity by an increase in the activity of adenyl cyclase, not a decrease in the activity of phosphodiesterase.

Adenyl cyclase is a multipass transmembrane protein with its catalytic domain on the cytosolic side. There are more than 8 isoforms in mammals and it is regulated by G-proteins and Ca^{2+}. All receptors that act via cAMP are coupled to stimulatory G proteins (G_s) which activate adenyl cyclase, thus increasing cAMP. Inhibitory G proteins (G_i) inhibit adenyl cyclase, by regulating ion channels, not by decreasing the cAMP content. cAMP can directly activate certain ion channels, but most of its effect is activating PKA. PKA catalyses transfer of the terminal phosphate group of ATP to specific serines/threonines of the target protein, thereby regulating their activity.

CRE is the cAMP response element. It is found in the regulatory region of many genes activated by cAMP. A binding protein, CREB recognises this sequence (Brindle & Montminy 1992). When CREB is phosphorylated by PKA on a single serine residue, it recruits a transcriptional co-activator, the CREB binding protein (CBP) which stimulates transcription of the genes. Serine/threonine protein phosphatases rapidly reverse the

effects of PKA. A summary of the events involved in the cAMP pathway are shown in *Figure 1.7.*

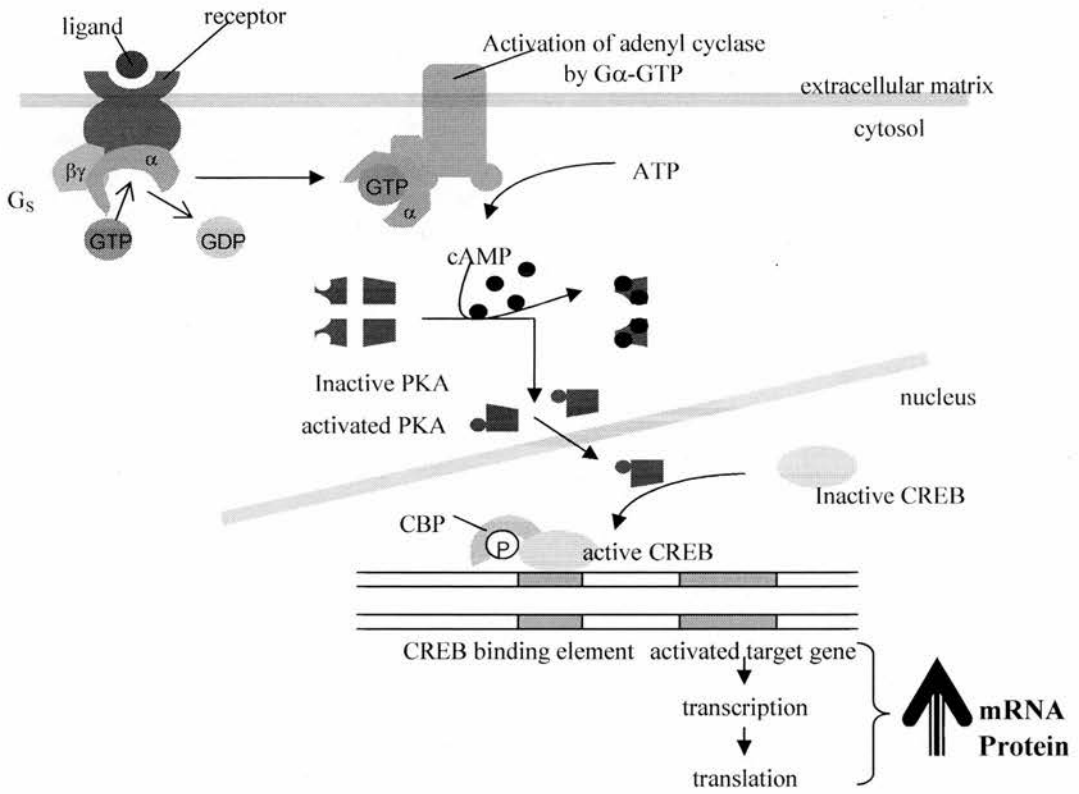


Figure 1.7: Summary of events involved in the cAMP/PKA pathway. Binding of the ligand to its receptor alters the receptor conformation, allowing binding of the G_s protein. This weakens the affinity of G_s for GDP in favour of GTP. Once GTP is bound, the α-subunit can dissociate, exposing its binding site for adenyl cyclase. The α-subunit binds to adenyl cyclase, activating it to hydrolyse ATP to cAMP. cAMP causes a conformational change in the regulatory subunit of PKA, causing dissociation of the subunits which become active. Active PKA phosphorylates CREB, which recruits CBP which stimulates transcription of target genes. Figure adapted from Alberts, Bray et al. 1994.

Agents, such as PGE_2 and β-adrenergic agents promote uterine quiescence via their ability to increase intracellular cAMP levels (Price & Bernal 2001). Elevated concentrations of cyclic AMP (cAMP) in the human myometrium may promote uterine quiescence during pregnancy by PKA-mediated phosphorylation and subsequent inactivation of myosin light-chain kinase, as well as by the phosphorylation and activation of cAMP-dependent transcription factors (Bailey et al 2000). It has been suggested that labour may be a result of the loss of this uterine quiescence. CREB has been identified in the myometrium, and levels are lower in pregnant non-labouring, than non-pregnant tissue (Bailey et al 2000). Adenyl cyclase has also been detected in both non-pregnant and pregnant human myometrium and changes in isoform expression identified during pregnancy. This consists of a shift from predominantly calcium-calmodulin stimulated isoforms, to those regulated by G-proteins (Price et al 2000). As well as its role in uterine quiescence, cAMP may also induce gene transcription of other proteins involved in labour, such as the MMPs (Lyons et al 2002).

1.5.8 Mitogen Activated Protein Kinase (MAPK) Pathways

The MAPK pathways are major signalling pathways in all eukaryotes and are involved in proliferation, differentiation and apoptosis (Kolch 2000, Kyosseva 2004). Ras proteins belong to a large superfamily of monomeric GTPases (Alberts et al 1994). They are signalling molecules and their activation is triggered by activated receptor tyrosine kinases. This is a short-lived response. Ras inactivates itself by hydrolysing its bound GTP to GDP via tyrosine specific protein phosphatases. When active, Ras activates a downstream serine/threonine phosphorylation cascade that includes a mitogen activated protein kinase (MAPK). Full activation of MAPK requires phosphorylation of a tyrosine and threonine residue. This is carried out by MAPK kinase (MEK) which is phosphorylated by a MEK kinase (Raf). It is Raf which is activated by Ras. The phosphorylated MAPK then relays the signal by phosphorylating proteins further downstream, including other protein kinases and gene regulatory proteins, thus causing regulation of gene expression and protein activity (*Figure 1.8*). There are 3 distinct

MAPKs in mammals: the extracellular signal-regulated kinases (ERKs), the c-Jun NH_2-terminal kinases (JNKs) and the p38 MAP kinases (Chang & Karin 2001).

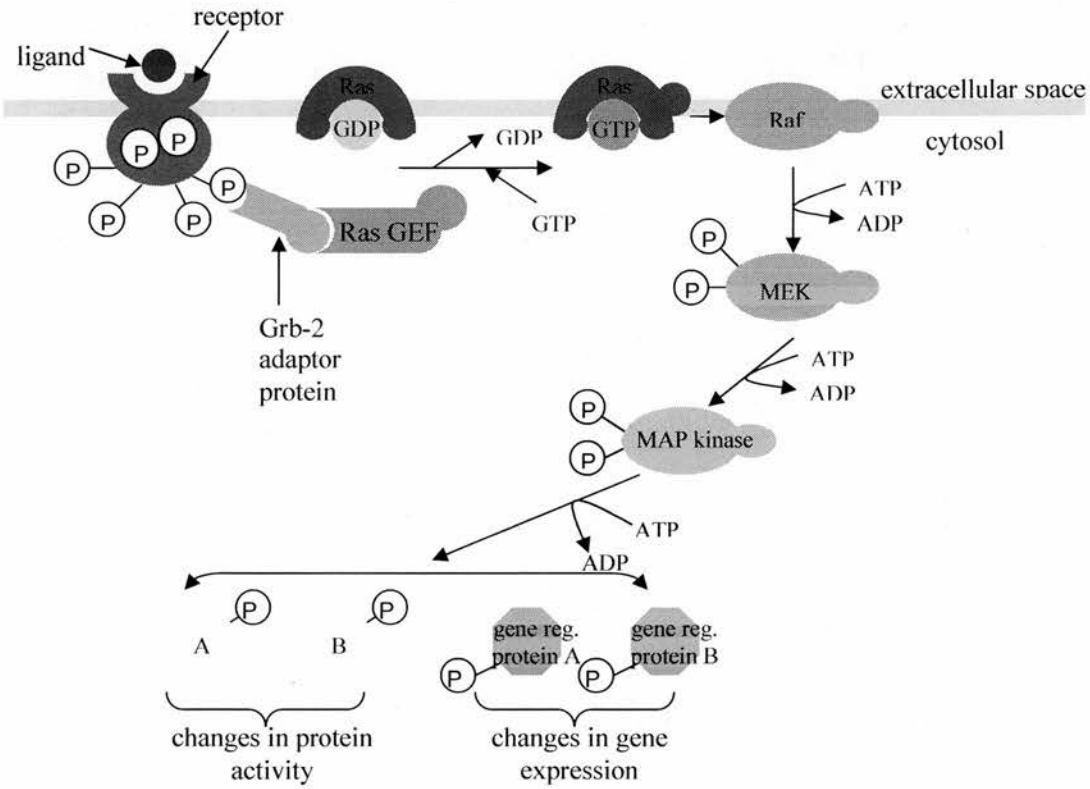


Figure 1.8: The mitogen activated protein kinase (MAPK) pathway of intracellular signalling. Phosphorylation of tyrosine activates Ras, which activates Raf, a serine/threonine kinase. This can phosphorylate MAPK kinase, MEK, which phosphorylates MAPK which ultimately leads to the regulation of gene expression and protein activity. Figure adapted from Alberts, Bray et al. 1994.

The MAPKs have been shown to play many roles in parturition. Studies show p38 MAPK is involved in the induction of COX-2 expression in the myometrium by IL-1β (Bartlett et al 1999). p38 MAPK and ERK are involved in the regulation of COX-2 expression by phospholipase D (Park et al 2002) and sphingosine-1 phosphate in amnion derived WISH cells (Kim et al 2003), respectively. p38 MAPK plays a role in decidual function at parturition (Takanami-Ohnishi et al 2001), and ERK is involved in the phosphorylation of myometrial caldesmon (an actin binding protein involved in smooth muscle contraction (Marston & Lehman 1985)) during pregnancy and labour (Li et al 2003). Both ERK and p38 MAPK, though not JNKs, have also been shown to mediate MMP-9 expression in vascular smooth muscle cells (Cho et al 2000).

1.5.9 Inositol Triphosphate Pathway

Some G proteins activate the inositol phospholipid signalling pathway by activating phospholipase-Cβ (Michell 1992). Phospholipase-Cβ acts on an inositol phospholipid (a phosphoinositide) called phosphatidylinositol 4,5-biphosphate (PIP_2) which is present in small amounts in the inner half of the plasma membrane lipid bilayer. Receptors acting this way activate a specific trimeric G protein, G_q, causing the α-subunit to dissociate and activate phospholipase-Cβ (*Figure 1.9*). The active phospholipase hydrolyses PIP_2 to inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG). The IP_3 is a small water soluble molecule which leaves the plasma membrane and diffuses rapidly into the cytosol. There it releases Ca^{2+} from the endoplasmic reticulum by binding to IP_3-gated Ca^{2+} release channels (Berridge 1993, Streb et al 1983). The IP_3 reponse is very short lived and can be terminated by de-phosphorylating and thus inactivating IP_3, and by pumping out the Ca^{2+}. The DAG has 2 potential signalling roles. It can be further cleaved to release arachidonic acid, which can be used as a messenger (for example to activate PKC (Blobe et al 1995), in a mechanism distinct from PKC activation by DAG (see below)), or in the synthesis of eicosanoids. Also, it can activate a serine/threonine protein kinase - protein kinase C (PKC) (Kishimoto et al 1980, Takai et al 1979). This is Ca^{2+}dependent, and it is thought that the initial rise in Ca^{2+} induced by IP_3 causes it to translocate from the cytosol to the cytoplasmic face of the plasma membrane. Here it is

activated by DAG, $Ca^{2+}$and phosphatidylserine and can phosphorylate cell proteins and alter gene transcription.

Inositol phosphates are involved in signaling at labour. Cytokines, such as IL-6, activate the IP_3 signalling pathway in the myometrium, causing cytosolic calcium oscillations that result in the development and maintenance of phasic myometrial contractions (Phillippe & Chien 1998). This occurs via the PLCγ isoform of PLC which is activated by a receptor tyrosine kinase, as opposed to GPCR in the case of PLCβ. Oxytocin increases IP_3 levels and may influence labour by stimulating PGE_2 production in amnion through the IP_3 pathway (Moore et al 1988). However, levels of IP_3 receptor in the human myometrium are similar in pregnant and non-pregnant tissue, and also remain constant during labour (Rivera et al 1990).

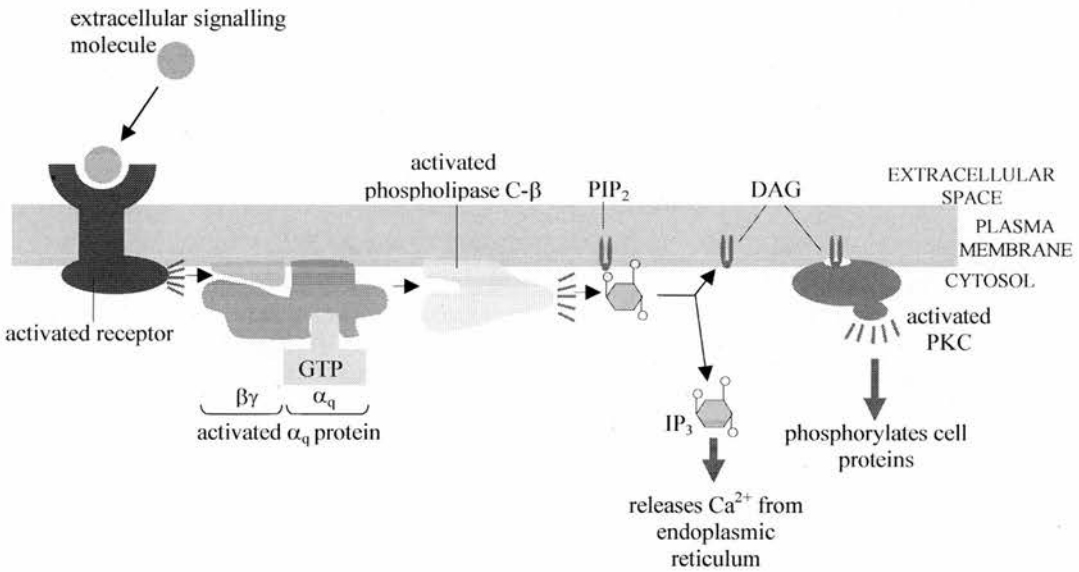


Figure 1.9: The 2 branches of the inositol phospholipid pathway. The activated receptor couples to G_q protein causing the α-subunit to dissociate and activate phospholipase C-β which cleaves PIP_2 to generate IP_3 and diacylglycerol. The DAG, along with Ca^{2+} and phosphatidylserine, activate PKC. Figure adapted from Alberts, Bray et al. 1994.

1.6 Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) constitute a multigene family of secreted and cell surface enzymes capable of degrading virtually every structural component of extracellular matrix (ECM) (Hulboy et al 1997). Other targets include other proteinases, proteinase inhibitors, clotting factors, chemotactic molecules, latent growth factors, growth-factor binding proteins, cell surface receptors and cell-cell adhesion molecules (Sternlicht & Werb 2001). They can thus regulate a wide variety of biological functions, including playing a range of roles in the human reproductive system. During pregnancy and parturition, there are many processes which involve extensive tissue remodelling, including implantation, cervical dilation, and rupture of the fetal membranes. A lot of this remodelling is influenced by the action of MMPs. MMP concentrations and activity can be regulated by reproductive hormones, as well as by cytokines and growth factors that participate in reproductive events. This section will deal with background information about MMPs, MMP structure and regulation, and the role of MMPs in reproductive processes and other biological events. The focus of MMP studies in this thesis is on the gelatinases, namely MMP-2 and MMP-9.

1.6.1 Identification of Matrix Metalloproteinases

Degrading extracellular proteins is essential for any individual cell to interact with its surroundings and for multicellular organisms to develop and function normally and it is extracellular proteinases that are responsible for this. In 1962 it was first shown that diffusible enzymes produced by fragments of involuting tadpole tail could degrade gels made of native fibrillar collagen by measuring degraded radioactive collagen (Gross & Lapiere 1962). Since then, a family of related enzymes has been identified and collectively named matrix metalloproteinases (MMPs) due to their dependence on metal ions for catalytic activity, their potent ability to degrade structural proteins of the extracellular matrix, and specific evolutionary sequence considerations that distinguish them from other closely related metalloproteinases of the metzincin superfamily, such as the astacins, serralysins and adamalysins (Stocker et al 1995). These metzincins are zinc

endopeptidases and are all distinguished by a highly conserved motif containing three histidines that bind zinc at the catalytic site. This signature motif is HEBXHXBGBXHZ where (H) histidine, (E) glutamic acid and (G) glycine are fixed, B is a bulky hydrophobic residue, X is a variable residue, and Z is a family-specific amino acid. The subfamily identity is based on this specific amino acid, which is serine in all but a few MMPs (Sternlicht & Werb 2001). In addition to cleaving proteins of the ECM, the MMPs act upon cell surface molecules and other pericellular non-matrix proteins, thus regulating cell behaviour in a number of ways (Sternlicht & Werb 2001). The MMPs play a role in many diverse physiological and pathological processes, including aspects of embryonic development, tissue morphogenesis, wound repair, inflammatory diseases, and cancer (Nelson et al 2000, Sternlicht et al 2000).

1.6.2 Nomenclature and Classification of MMPs

To date, around 25 MMPs have been identified in humans and other vertebrates in addition to several non-vertebrate MMPs. They are generally referred to by their common name or according to a sequential numeric nomenclature for the vertebrate MMPs. They can be grouped as to their specific substrates also. These MMPs are classified in *Table 1.3*.

1.6.3 Structure of MMPs

Nearly a decade after collagenolytic activities were first discovered, it was found that MMPs are synthesised and secreted as inactive zymogens (pro-MMPs) that require activation (Harper et al 1971). This pro-domain contains a unique sequence, including a cys, known as the cysteine switch, which ligates the catalytic zinc to maintain the latency of pro-MMPs (Becker et al 1995, Van Wart & Birkedal-Hansen 1990). There is a catalytic domain containing a zinc binding motif and a conserved methionine, which forms a unique 'Met-turn' structure (Bode et al 1993). The domain consists of a five-stranded β-sheet, three α-helices and bridging loops (Dhanaraj et al 1996). These backbones structures, including the Met-turn are similar to those of other metalloproteinases (Bode et al 1993). The MMPs additionally have a structural zinc ion,

Group	MMP	Common Name	MW (kDa) Latent (active)	Substrates
Collagenases	MMP-1	Interstitial collagenase	52 (42)	Collagens I, II, III, VI, X, gelatins
	MMP-8	Neutrophil collagenase	75 (58)	Collagens I, II, III, aggrecan
	MMP-13	Collagenase-3	60 (48)	Collagens I, II, III
	MMP-18	Collagenase-4		?
Gelatinases	MMP-2	Gelatinase-A	72 (66)	Gelatins, collagens I, IV, V, VII, X, XI, fibronectin, laminin, aggrecan, Elastin,
	MMP-9	Gelatinase-B	92 (86)	Gelatins, collagens IV, V, XIV, aggrecan, elastin,
Stromelysin	MMP-3	Stromelysin-1	57 (45)	Aggrecan, gelatins, fibronectin, laminin, collagen II, IV, IX, X
	MMP-10	Stromelysin-2	57 (44)	Aggrecan, fibronectin, laminin, collagen IV
	MMP-11	Stromelysin-3	51 (44)	Fibronectin, laminin, collagen IV, aggrecan, gelatins
Membrane-type MMPs	MMP-14	MT-MMP-1	63 (?)	Collagens I, II, III, fibronectin, laminin, proteoglycan, ProMMP-2, ProMMP-13
	MMP-15	MT-MMP-2		?
	MMP-16	MT-MMP-3		ProMMP-2
	MMP-17	MT-MMP-4		?
	MMP-24	MT-MMP-5		
	MMP-25	MT-MMP-6		
Other	MMP-7	Matrilysin, Pump-1	28 (19)	Aggrecan, fibronectin, laminin, collagen IV, elastin
	MMP-26	Endometrase, Matrilysin-2		
	MMP-12	Metalloelastase	54 (45/22)	Elastin, plasminogen
	MMP-19	RASI-1		
	MMP-20	Enamelysin	54	amelogenin
	MMP-21	XMMP (Xenopus)		
	MMP-22	CMMP (chicken)		
	MMP-23	CA-MMP		
	MMP-27	CMMP (Gallus)		
	MMP-28	Epilysin		

Table 1.3: MMP classification (adapted from Sternlicht and Werb 2001).

and two to three calcium ions which are required for the stability and expression of enzymatic activity. The gelatinases have three-repeats of fibronectin-type II in the catalytic domain which interact with collagens and gelatin (Allan et al 1995, Steffensen et al 1995). The catalytic domain shows cleavage specificity through its active site cleft, through specificity sub-site pockets that bind amino acid residues immediately adjacent to the scissile peptide bond, and through secondary substrate-binding exosites located outside the active site itself (Overall 2001). A proline-rich linker peptide joins the catalytic domain to the C-terminal hemopexin domain. The function of this is not known although it may adopt a collagen-like conformation which can disturb the quaternary organisation of the triple-helix in the collagenase-susceptible site (De Souza et al 1996). The hemopexin domain is essential for collagenases to cleave triple helical interstitial collagens (Bode 1995) although the catalytic domains alone retain proteolytic activity towards other substrates (Clark & Cawston 1989). All MMPs have an N-terminal 'pre' domain that is removed after it directs their synthesis to the endoplasmic reticulum. Most MMPs are secreted, however, the membrane type MMPs have transmembrane domains and are expressed as cell surface enzymes. A representation of the domain structures of different MMP groups is shown in *Figure 1.10*. The gelatinases, MMP-2 and MMP-9 are distinguished from other MMPs by a fibronectin type II repeat insert that mediates their ability to bind to and degrade gelatine and other components of the ECM (Shipley et al 1996). The gelatinases have similar substrate specificities, but differ in terms of their regulation at many levels.

1.6.4 Regulation of MMPs

In order for MMPs to carry out their roles, they must be localised to the correct cell type at the correct time and activated or inhibited suitably. Thus, MMPs are controlled at a transcriptional and post-transcriptional level and at a protein level via secretion, activation, inhibition, localisation and their removal (Sternlicht & Werb 1999).

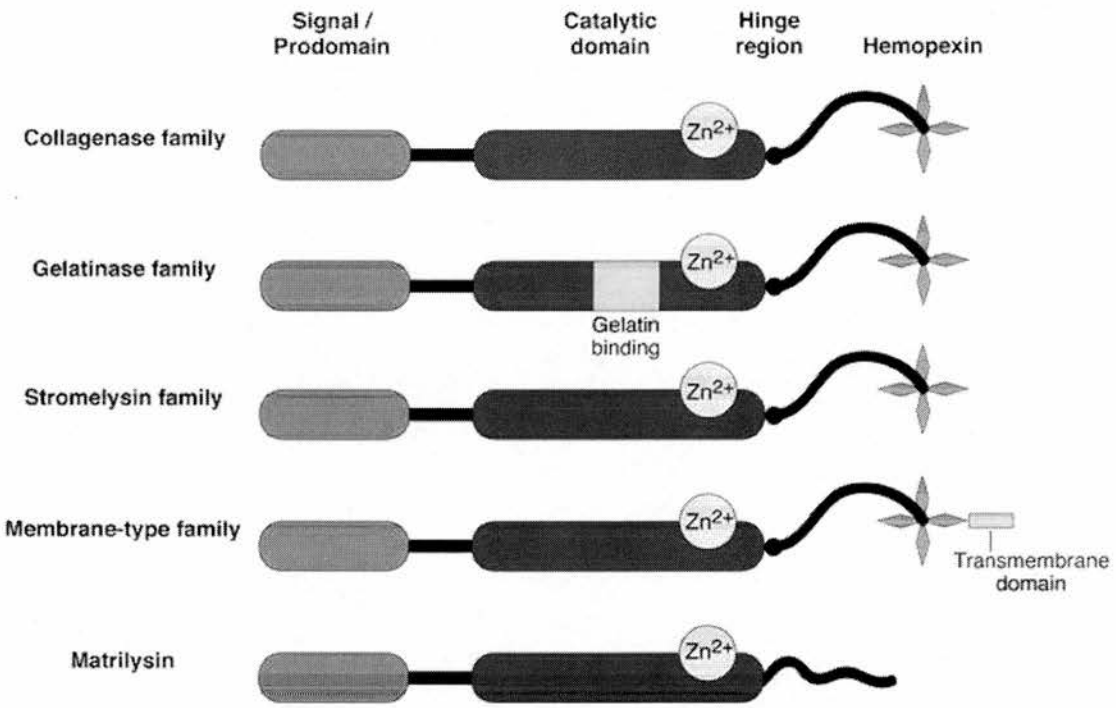


Figure 1.10: Domain structure of MMPs (adapted from Calbiochem "Extracellular Matrix Proteases and Protein Technical Guide", Volume 2, 2002)

1.6.4.1 Transcriptional Control of MMPs

With the exception of MMP-2, which is often constitutively expressed and controlled through enzyme activation (Strongin et al 1995), and post-transcriptional mRNA stabilisation (Overall et al 1991), MMPs are closely regulated at the level of transcription by numerous stimulatory and suppressive factors that influence multiple signalling pathways. The effectors include growth factors, cytokines, endocrine factors such as steroids, chemical agents (e.g. phorbol esters which work by activating PKC as they resemble DAG), extracellular matrix proteins, physical stress and changes in cell shape (Kheradmand et al 1998). MMP-11 is only transcriptionally activated by retinoic acid (Guerin et al 1997), or by stromal cell interactions with tumour cells (Ahmad et al 1997).

Activating Protein-1 (AP-1) sites, which bind the Fos and Jun families of transcription factors, and Polyoma Enhancer A-binding Protein-3 (PEA-3) sites, which bind the Ets family of transcription factors and are present in the promoters of inducible MMP genes. These are essential for transcriptional activation. MMP-1 contains adjacent AP-1 and PEA-3 sites that are proximal to the transcriptional start site, and these sites are required for activation of the promoter by transforming oncogenes and tumour promoting phorbol esters (Vincenti et al 1998). The proximal AP-1 site contributes to basal MMP-1 transcription, whereas a second AP-1 site, located further 5' in the promoter, mediates transcriptional induction by phorbol esters (White & Brinckerhoff 1995). Other transcription factors often contribute, particularly in response to growth factors and cytokines. IL-1 transactivates the rabbit MMP-1 promoter through an NF-κB binding site near the transcription start site, which cooperates with the proximal AP-1 site. This cooperativity between NF-κB and AP-1 also activates the MMP-9 promoter in response to TNF-α (Sato & Seiki 1993) or IL-1 (Yokoo & Kitamura 1996).

1.6.4.2 Post Transcriptional Regulation of MMPs

Post transcriptional mechanisms are also a means by which regulation of many MMPs occurs. It has been suggested that multiple transcripts of MMP-13, MMP-17 and MMP-

20 probably result from alternative polyadenylation (Bartlett et al 1996, Freije et al 1994, Puente et al 1996). Also, a soluble and proteolytically active form of MT3-MMP (SM3) is generated by alternative splicing (Matsumoto et al 1997a). MMP-1 and MMP-3 mRNA are stabilised by phorbol esters and EGF, and MMP-13 transcripts are stabilised by PDGF and glucocorticoids and destabilised by TGF-β (Delany et al 1995, Vincenti 2001). AU-rich sequences are also required for regulated decay of MMP-1 transcripts (Vincenti 2001).

1.6.4.3 Secretion of MMPs

Once translated, most MMPs are constitutively secreted, however some regulation does occur at this level. MMP-8 and MMP-9 are synthesised by differentiating granulocytes in the bone marrow, stored in the specific and gelatinase (tertiary) granules or circulating neutrophils, respectively, and released following neutrophil activation by inflammatory mediators (Hasty et al 1990). In macrophages, post-translational release of MMP-12 occurs in response to PKC activation downstream of a G-protein-coupled thrombin receptor. This becomes activated after binding a ligand that is generated by its own N-terminal thrombin-dependent cleavage (Raza et al 2000).

1.6.4.4 Localisation of MMPs

Expression of some MMP genes is cell specific. For example, three interstitial collagenases, MMP-1, MMP-8 and MMP-13 have similar substrate specificities in that they all degrade collagens I, II, and III. However, their cellular expression is different, suggesting that they play distinct roles in tissue remodelling. MMP-1 is most broadly expressed, and can be induced in fibroblasts, chondrocytes, macrophages, endothelial cells and keratinocytes (Vincenti et al 1996). MMP-8 is stored in neutrophils as granules that are released upon cellular activation. MMP-13 was first cloned from human breast carcinomas (Freije et al 1994) and has since been shown to be expressed in other carcinomas (Johansson et al 1997).

Localisation of MMP protein within the cell is also important. It appears that cell surface localisation can control the activity of the MMP. The mechanisms for localising MMPs to the cell surface and to specific cell surface subdomains include the expression of membrane-bound MMPs, binding of MMPs to cell surface receptors, presence of cell surface receptors for MMP-activating enzymes such as uPA, plasminogen, thrombin and elastase, and the concentration of MMPs on pericellular ECM molecules. This localisation can enhance MMP activation, limit access of MMP inhibitors, concentrate MMPs within the vicinity of their targets, and limit the extent of proteolysis to discrete pericellular regions (Sternlicht & Werb 2001). Cell surface docking is also important in providing a mechanism to tether MMP activity to the regions of physical contact between the cell and ECM, where ECM remodelling occurs. This provides a means to control ECM degradation (Stamenkovic 2003). The localisation of the MMPs within the ECM is also important.

1.6.4.5 Activation of MMPs

Like other proteolytic enzymes, most MMPs are synthesised as inactive pro-enzymes. The cysteine sulph-hydryl group in the propeptide domain acts as a fourth ligand for the active site zinc ion, and activation requires that this cysteine switch be opened by normal proteolytic removal of the pro-peptide domain, or by ectopic perturbation of the cysteine-zinc interaction (Van Wart & Birkedal-Hansen 1990). Once displaced, the thiol group is replaced by a water molecule that can then attack the peptide bonds of MMP targets (*Figure 1.11a*). Secreted pro-MMPs can be activated in-vitro by proteinases and by non-proteolytic agents such as SH-reactive agents, mercurial compounds, reactive oxygen and denaturants. In vivo, most pro-MMPs are likely to be activated by plasma proteinases or opportunistic bacterial proteinases.

Some MMP members are not secreted as inactive zymogens, including MMP-11, MMP-27 and MT-MMPs. They contain a furin-like enzyme recognition motif between their propeptide and catalytic domains. This allows them to be activated by intracellular subtilisin-type serine proteinases before they reach the cell surface or are secreted

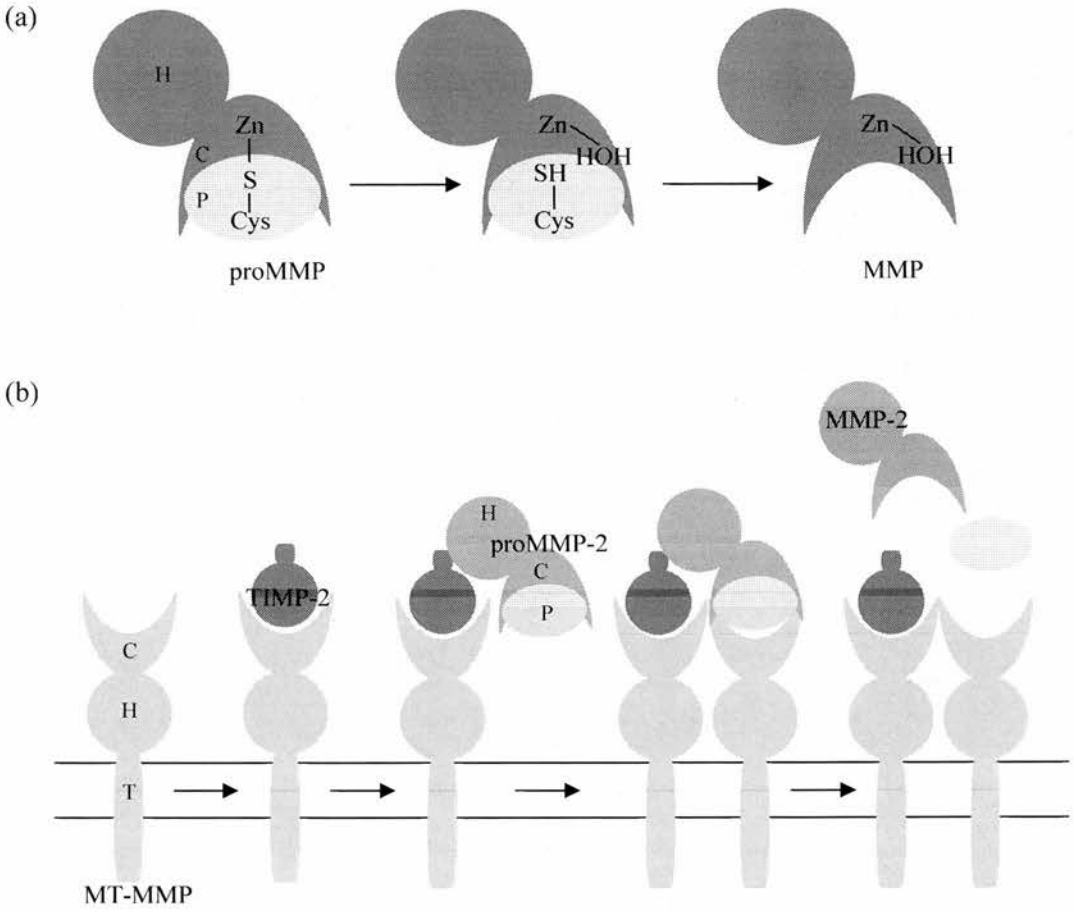


Figure 1.11: (a) General activation of most latent MMPs. The latency of the proMMP is maintained by an unpaired cysteine sulphhydryl group in the propeptide domain. The sulphydryl group acts as a fourth ligand for the zinc in the catalytic domain. During activation, this cysteine is displaced from the complex with removal of the propeptide domain and is replaced by a water group. (b) Activation of latent MMP-2. A membrane type MMP (MT-MMP) is inhibited by a tissue inhibitor of metalloproteinases (TIMP-2). The hemopexin domain of proMMP-2 then binds to the C-terminal portion of TIMP-2. An uninhibited MT-MMP then removes the propeptide domain. C = catalytic domain, H = hemopexin domain, P = propeptide domain, T = transmembrane domain. Figures adapted from Visse & Nagase 2003.

(Pei & Weiss 1995). MMP-23, which lacks the cysteine switch of other MMPs which is required for latency (Gururajan et al 1998), has a furin-susceptible cleavage site and is a likely target of intracellular proprotein convertases. All other MMPs lack a furin-susceptible insert and are thus activated outside the cell following secretion.

Activation of most MMPs can be initiated by already active MMPs or by serine proteases which cleave peptide bonds within MMP pro-domains (Sternlicht & Werb 2001). Activation of MMP-2 is different (*Figure 1.11b*). It is activated at the cell surface in a process involving MT-MMPs and TIMP-2 (Strongin et al 1995). During this process, an active MT-MMP is inhibited by TIMP-2. The hemopexin domain of pro-MMP-2 then binds to the C-terminal portion of TIMP-2 to form a tri-molecular complex. A free, uninhibited MT-MMP then partially activates the pro-MMP-2 by removing most of the propeptide. The remaining portion of propeptide is removed by a separate MMP-2 molecule at the cell surface, giving fully active MMP-2. This can then be released from the cell surface, or bind to another MMP-2 docking protein. It seems that MT1-MMP is an efficient activator of MMP-2, whereas MT2-MMP and MT4-MMP cannot activate MMP-2 (Sternlicht & Werb 2001).

1.6.4.6 Inhibition of MMPs

1.6.4.6.1 Tissue Inhibitors of Metalloproteinases (TIMPs)

The activity of secreted MMPs is restricted by endogenous tissue inhibitors of metalloproteinases (TIMPs). The TIMPs consist of at least 4 members which have been identified in vertebrates to date (Gomez et al 1997) as shown in *Table 1.4*.

The TIMPs share between 37 and 51 % sequence homology and a conserved gene structure. They have an N- and C-terminal domain of about 125 and 65 amino acids respectively, each one containing three disulfide bonds (Murphy et al 1991, Williamson et al 1990). The N-terminal domain folds into a separate unit and interacts with the MMP catalytic site (Murphy et al 1991, Murphy & Willenbrock 1995). Mutational analysis (Huang et al 1997, O'Shea et al 1992, Willenbrock & Murphy 1994) and

TIMP	Mass (kDa)	Extracellular location	Reported function
TIMP-1	30	Soluble in ECM and body fluids	Inhibits all known MMPs, associates with pro-MMP-9, inhibits angiogenesis
TIMP-2	21/28	Soluble in ECM and body fluids	Inhibits all known MMPs, associates with MT1-MMP and MMP-2 at cell surface, regulates MMP-2 activation
TIMP-3	24 (28)	Bound to ECM	Inhibits all known MMPs. Mutation linked with Sorsby's fundus dystrophy
TIMP-4	29		Inhibits all known MMPs. Restricted expression suggests tissue specific TIMP function

Table 1.4: TIMPs: classification, molecular weights and role in the ECM.

peptide and anti-body blocking experiments (Bodden et al 1994) have helped to identify which regions of the N-terminal are involved in MMP inhibition. Analysis by NMR first identified the N-terminal domain structure of TIMP (Williamson et al 1994), then X-ray crystallographic studies of TIMP-1-MMP-3 complexes (Gomis-Ruth et al 1997) and TIMP-2-MT1-MMP (Fernandez-Catalan et al 1998) revealed the inhibition mechanism. The TIMP molecule slots into the active cleft of an MMP molecule, much like a substrate would. The TIMP and MMP bind with a 1:1 stochiometry and kinetic experiments indicate that interactions first form a reversible intermediate complex, followed by rearrangement to a stable inhibitory complex (Hutton et al 1998). Although most inhibitory activity is in the N-terminal, both domains influence enzyme-inhibitor binding (Willenbrock & Murphy 1994). The C-terminal domain of TIMP-1, for example, binds more favourably to the hemopexin domain of MMP-9 than that of MMP-2, whereas the C-terminal of TIMP-2 prefers to bind the hemopexin domain of MMP-2 (Morgunova et al 2002, Murphy & Willenbrock 1995).

The high level of sequence divergence between the four TIMPs suggests that they probably have different functional properties. Although they all bind tightly to most

MMPs (Murphy & Willenbrock 1995), inhibitory abilities are variable. TIMP-2 and TIMP-3 are good inhibitors of the MT-MMPs while TIMP-1 is not, and TIMP-3, but not the others, is a good inhibitor of TNF-α converting enzyme (Amour et al 1998), and other members of the ADAM family (a disintegrin and metalloproteinase) (Amour et al 2000, Kashiwagi et al 2001, Loechel et al 2000). TIMPs also vary in their patterns of gene regulation and tissue distributions.

1.6.4.6.2 Other Endogenous Inhibitors

Another class of MMP inhibitors, the protein subdomains, have structural similarity to the TIMPs. Proteolytic processing of the procollagen C-terminal proteinase enhancer protein, PCPE, releases a C-terminal fragment (CT-PCPE) that has structural similarity and MMP inhibitory activity to the N-terminal domain of the TIMPs (Mott et al 2000). There are also sequence similarities between TIMPs and the noncollagenous C-terminal domain of type IV collagen and functional analysis shows that this also has MMP inhibitory activity (Netzer et al 1998) and can inhibit tumour growth and angiogenesis (Petitclerc et al 2000). It is unclear whether MMPs are the main target of these domains, despite CT-PCPE having a much higher inhibiting affinity for MMP than TIMP-2 (Mott et al 2000).

1.6.4.7 Catabolism and Clearance

Although much progress has been made in understanding proteolytic processing of pro-MMPs, relatively little is known about the further proteolysis of active MMPs. Some cleavages inactivate MMPs, whereas others, for example a cleavage to specifically remove the C-terminal, hemopexin domain can generate a truncated form of the enzyme, which loses its ability to act on some substrates but not others. This can diminish their affinity and ability to be inhibited by TIMPs (Itoh et al 1998). Removal of the hemopexin domain also removes the ability of some MMPs to localise to the cell surface. MT-MMPs can be secreted if they are cleaved at a juxtamembrane site before or after they reach the cell surface (Imai et al 1996). So, factors which influence MMP

degradation also alter the steady-state concentrations of MMPs, their substrate specificities, their localisation and their ability to be activated and inactivated.

MMPs can also be regulated by direct clearance of intact enzymes. Most MMPs cleave α2-macroglobulin, initiating a conformational change in it that irreversibly traps the enzyme (Sottrup-Jensen & Birkedal-Hansen 1989). This physical entrapment keeps the enzyme from acting on its substrates and eventually the complex of α2-macroglobulin and the MMP is endocytosed and permanently cleaved.

1.6.5 Role of MMPs in Tissue Remodelling of the ECM During Pregnancy and Parturition

MMPs play a key part in tissue remodelling of extracellular matrix of uterine and gestational tissues throughout pregnancy and parturition. As discussed previously, they are involved in tissue remodelling of the cervix during cervical ripening and dilation, and rupture of the fetal membranes. A summary of the role of MMPs in tissue remodelling and rupture of the membranes is shown in *Figure 1.12*. It is suggested that an inflammatory cascade causes the ratio of MMP:TIMPs to increase by a combination of MMP levels increasing, and TIMP levels decreasing. This increases degradation of collagen, weakening the fetal membranes, leading to eventual rupture. MMPs are also involved in tissue remodelling at the implantation stage of pregnancy, and establishment of the placenta.

1.6.6 Roles of MMPs in Other Biological Systems

MMPs are able to regulate numerous biological processes besides their effects in the reproductive system. They may promote the initial stages of cancer development, but may also decrease the severity of the ultimate malignancy (Coussens et al 2000, Pozzi et al 2000). In arthritis, the loss of certain MMPs aggravates, rather than alleviates the disease (Mudgett et al 1998). MMP-9 has also been implicated in β-amyloid degradation in Alzheimer's disease (Helbecque et al 2003).

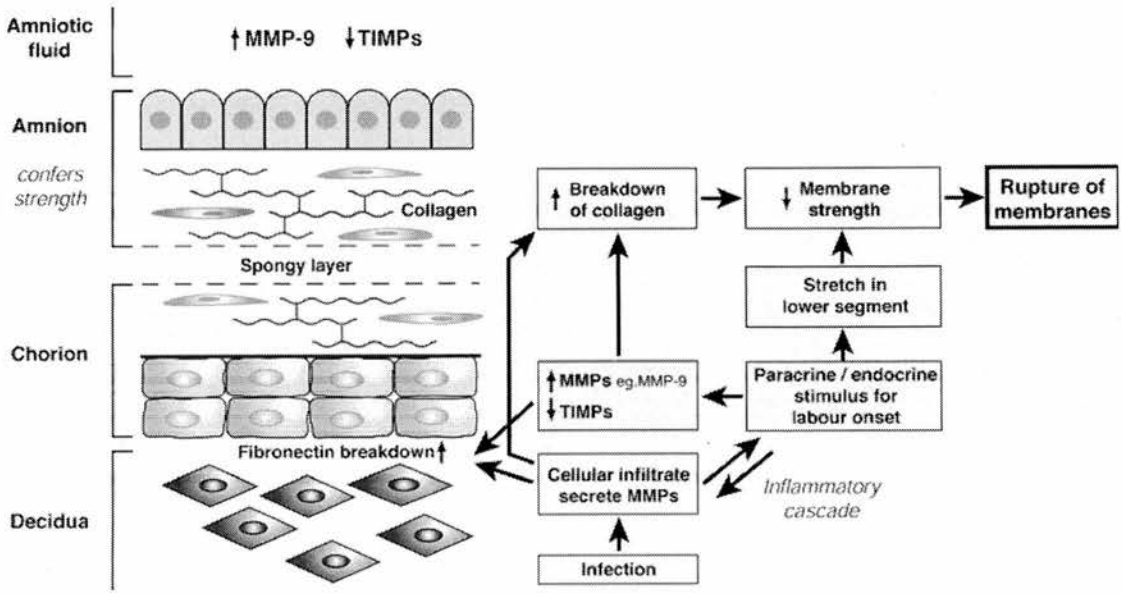


Figure 1.12: Role of MMPs and their inhibitors, TIMPs in tissue remodelling of the fetal membranes and their contribution to rupture of the membranes.

1.7 Aims and Hypothesis

It is well known that the prostaglandins PGE_2 and $PGF_{2\alpha}$ play a role in many aspects of labour. Previous studies have localised the enzymes involved in prostaglandin synthesis and catabolism within intrauterine and gestational tissue. So far, receptors for PGE_2 and $PGF_{2\alpha}$ have been identified in the human myometrium, and the decidua of the baboon. There are no published reports of these receptors being localised in the fetal membranes, decidua or placenta in the human. In this thesis, the presence and localisation of the EP2, EP4 and FP receptors within these tissues will be investigated. Furthermore, samples will be examined from preterm and term samples, in both spontaneous labour and non-labour cases and localisation within the tissues compared.

The signalling pathways via the EP2, EP4 and FP receptors have been well characterised in other tissues, including the endometrium. EP2 and EP4 are coupled to stimulatory G-proteins and activation causes elevation in cAMP levels and activation of the PKA pathway. The FP receptor is coupled to a $G_{\alpha q}$ receptor and activation of this can elevate IP_3 levels via activation of phospholipase C, intracellular calcium flux and activation of protein kinase A. EP2, EP4 and FP receptors are also linked to Ras and activation of the MAP kinase pathway. This thesis deals with activation of the EP2, EP4 and FP receptors within the human amnion and chorio-decidua and the intracellular pathways which are stimulated. Specifically, the cAMP pathway, the MAPK pathway and the IP_3 pathway will be investigated. The JEG3 choriocarcinoma cell-line will also be used as a model of chorion trophoblast cells to investigate specific intracellular signalling within these cells.

Activation of intracellular signalling pathways can influence transcription of certain genes within the nucleus. Previous studies have shown that PGE_2 and $PGF_{2\alpha}$ can elevate MMP-2 and MMP-9 levels, and activation of MMP-2 within the human fetal membranes (McLaren et al 2000b, Ulug et al 2001). In this thesis, these findings of other research groups will be further investigated. Furthermore, the JEG3 cell-line will be used as a model of chorion trophoblast cells to investigate the effects of PGE_2, $PGF_{2\alpha}$ and IL-6, on MMP-2 and MMP-9 production and activation.

The aims of this thesis therefore are to:

a) characterise the distribution of prostaglandin receptors in human uterine and gestational tissue and investigate the effect of labour at term and preterm on this distribution.
b) investigate the signalling pathways by which prostaglandins act via these receptors.
c) examine the effects of prostaglandins on production and activation of MMPs.

The experimental work in this thesis aims to examine the hypothesis that EP2, EP4 and FP receptors are located in the human fetal membranes and binding of PGE_2 and $PGF_{2\alpha}$

can activate intracellular signalling pathways by elevating cAMP, IP_3 and MAPK levels. These influence events further downstream causing increases in transcription of the genes for MMP-2 and MMP-9 (*Figure 1.13*). The resulting increase in MMP levels contributes to rupture of the fetal membranes, thus demonstrating that PGE_2 and $PGF_{2\alpha}$ can lead to rupture of the fetal membranes and PPROM.

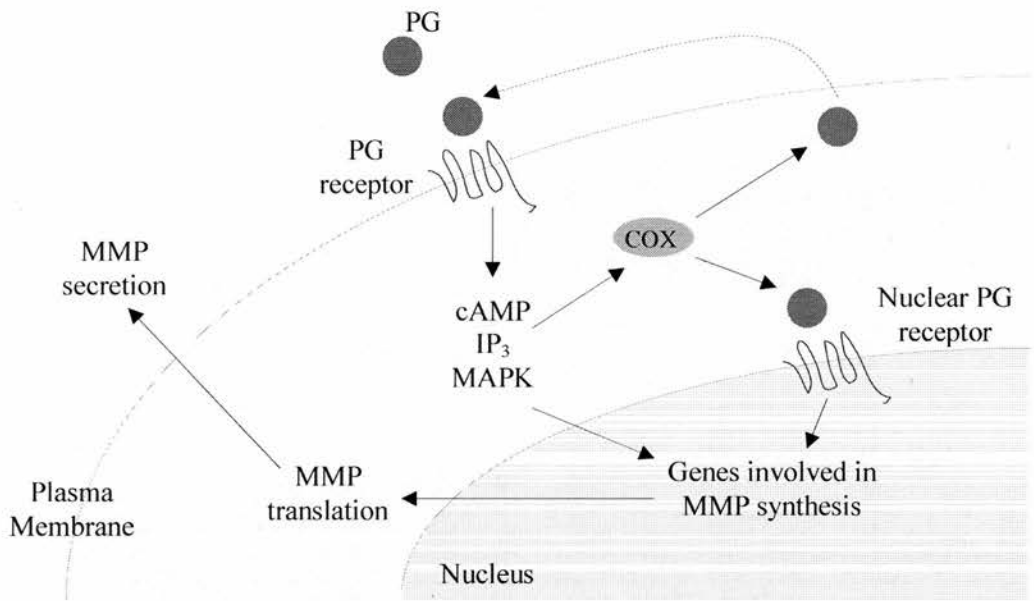


Figure 1.13: Hypothesised model for PGE_2 and $PGF_{2\alpha}$ actions in the fetal membranes. PG; prostaglandin, cAMP; adenosine-3',5'-cyclic monophosphate, IP_3; inositol triphosphate, MAPK; mitogen activated protein kinase, MMP; matrix metalloproteinase

Chapter 2: Methods

2.1 Tissue Collection

2.1.1 Fetal Membranes and Placental Tissue for Explants

Fetal membranes and placenta were collected from women with uncomplicated pregnancies at term not in labour immediately after elective caesarean section. Tissue was collected in DPBS containing 10 U/ml heparin. Women with clinical evidence of infection, multiple pregnancy, dysfunctional labour, or women who had received prostaglandin or artificial oxytocin were excluded. Collection of tissue was approved by the Lothian Research Ethics Committee (LREC; LREC no. 2002/6/27). Written, informed consent was obtained from all patients prior to tissue collection.

2.1.2 Tissue for Immunohistochemistry

At the time of delivery, the following tissues were collected from elective caesarean sections and emergency caesarean sections during active labour (defined as a cervical dilation of over 4 cm) for failure to progress: a cross-section of placenta from placental plate to decidua basalis from a centrally located cotyledon; decidua parietalis from the uterine aspect of the fundus; fundal myometrium; and fetal membranes from the fundal region remote from the placental edge. A clip was attached to the membranes overlying the internal os of the cervix allowing for orientation. Tissues were fixed in 10 % neutral-buffered formalin (appendix 1.1.1) for 24 hours, washed in 70 % ethanol, followed by 100 % ethanol, before being embedded in paraffin.

2.2 Tissue Culture

2.2.1 Amnion, Chorio-decidua and Placenta Explant Culture Methods

Fetal membranes and placenta collected after delivery were washed 5 times with DPBS. The amnion and chorion were then separated and these and the placenta steeped in steeping buffer (appendix 1.1.2) for 30 min at room temperature. These were then washed 5 times with DPBS. Discs of amnion (8 mm diameter) and chorio-decidua (6 mm diameter) were cut using a cork borer. Pieces of villous placental tissue were

dissected from the middle central cotyledon (wet weight 30-50 mg). Explants were placed on absorbent capillary matting in a 24-well plate and maintained in complete serum-free RPMI 1640 (appendix 1.1.3) for 24 hours at 37 °C in 95 % water-saturated air-5 % CO_2 atmosphere. Culture media was harvested and frozen at –20 °C until analysis.

2.3 Cell Culture

The JEG3 choriocarcinoma cell line (*Figure 2.1*) is one of 6 clones derived from human cells implanted into a hamster cheek pouch and propagated on irradiated feeder layers of human fibroblasts (Kohler & Bridson 1971). This cell-line secretes chorionic gonadotrophin, somatomammotrophin (placental lactogen) and progesterone. It is also able to transform steroid precursors to oestrone and oestradiol. There are no publications involving the expression and function of prostaglandin receptors EP and FP in this cell line. However, it has been shown that the receptor for 15-deoxy-$\Delta^{12,14}$-PGJ2, peroxisome proliferator activated receptor-γ (PPARγ) is expressed and functionally active in these cells (Marvin et al 2000). MMP-26 and TIMP-4 have been detected in these cells (Zhang et al 2002) and also MMP-2 and MMP-9 (Mandl et al 2002). The MAPK pathway has been investigated and found to be activated by fibronectin and vascular endothelial growth factor (VEGF) in these cells (Zhang et al 2003). It is considered therefore that these cells are a good model to investigate trophoblast cell function.

The JEG3 cell-line was maintained in complete DMEM with serum (appendix 1.1.4) in a 75 cm^3 flask and split 1 in 4 twice a week when the cells had reached confluence. Old medium was aspirated from the flask, the cells washed twice with 10 ml DPBS, then 1.5 ml of 0.5x trypsin/EDTA was added. When the cells became detached from the flask wall, 15 ml DMEM containing serum was added to quench the action of trypsin. Cells were then diluted 1 in 4 and put back in flasks to grow and maintain a stock. At this stage, cells could also be counted and plated out for use in experiments. Cells were counted using a haemocytometer, using trypan blue (appendix 1.1.5) to check the

viability of the cells. They were then diluted accordingly and plated out at a concentration of 2 x 10^5 cells/ml.

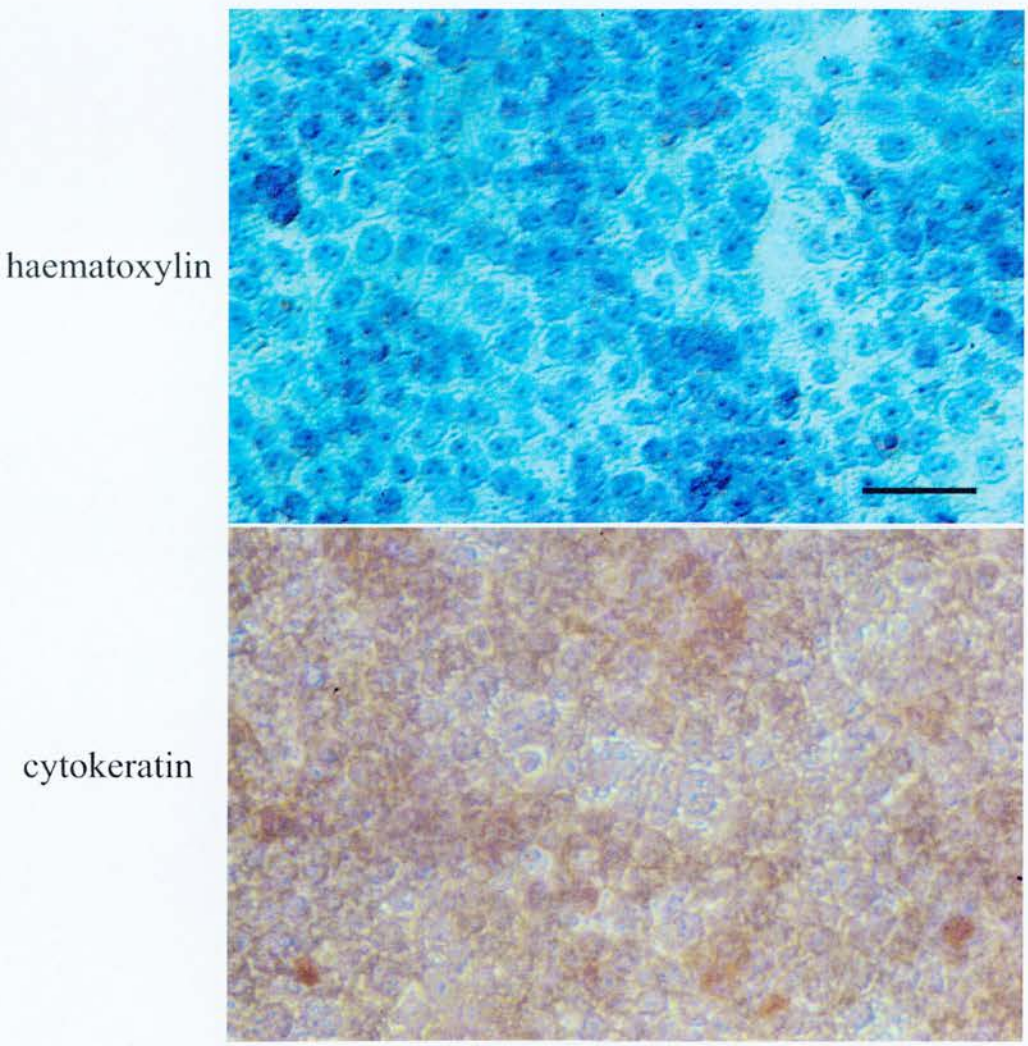


Figure 2.1: Image of JEG-3 cells visualized with haematoxylin and cytokeratin. Positive cytokeratin staining shows that the cells are of epithelial origin. Both photomicrographs are taken at the same magnification. The scale bar represents 50 μm.

2.4 Immunohistochemistry

Immunohistochemistry involves localisation of an antigen within tissue or cells by a specific primary antibody. Here we used the standard avidin-biotin peroxidase (ABC) to visualise specific staining (*Figure 2.2*). Primary antibody binds to the target antigen within the sample. A biotinylated secondary antibody, which is specific to the species the primary antibody was raised in, is then added which binds to the primary antibody. Addition of an avidin-biotin and horseradish-peroxidase complex results in any free sites on the avidin molecule binding to the biotin on the secondary antibody. When 3,3-diaminobenzidine (DAB) is added, it becomes oxidised. The resulting colour change of the chromagen causes an insoluble brown precipitate to form at the site of the protein of interest.

Non-specific staining may occur due to a variety of reasons. The primary antibody may bind non-specifically to other epitopes. If this is the case, the results can be compared to those obtained for Western Blotting to ensure they are valid. Tissues may contain endogenous peroxidase activity which can catalyse the DAB, causing brown staining to appear at the site of this endogenous activity. To counteract this, a weak solution of hydrogen peroxide was added to saturate any endogenous peroxidase, rendering it inactive. The secondary antibody may too be non-specific. This can cause it to bind to epitopes in the tissue mimicking those from species it was raised against, as well as the primary antibody. To stop this, normal serum from the species from which the secondary antibody was raised was added prior to addition of the primary antibody.

Inability of an antibody to detect a specific epitope must also be recognised as a potential problem when using this technique. The appropriate concentrations of primary antibody must be determined, and also the detection system validated. The epitope may be masked, for instance, by fixation. Different fixatives may be tested, and also antigen retrieval methods, for example boiling/microwaving sections or pre-treating with a

proteinase. The use of a positive control is also important if negative results are obtained.

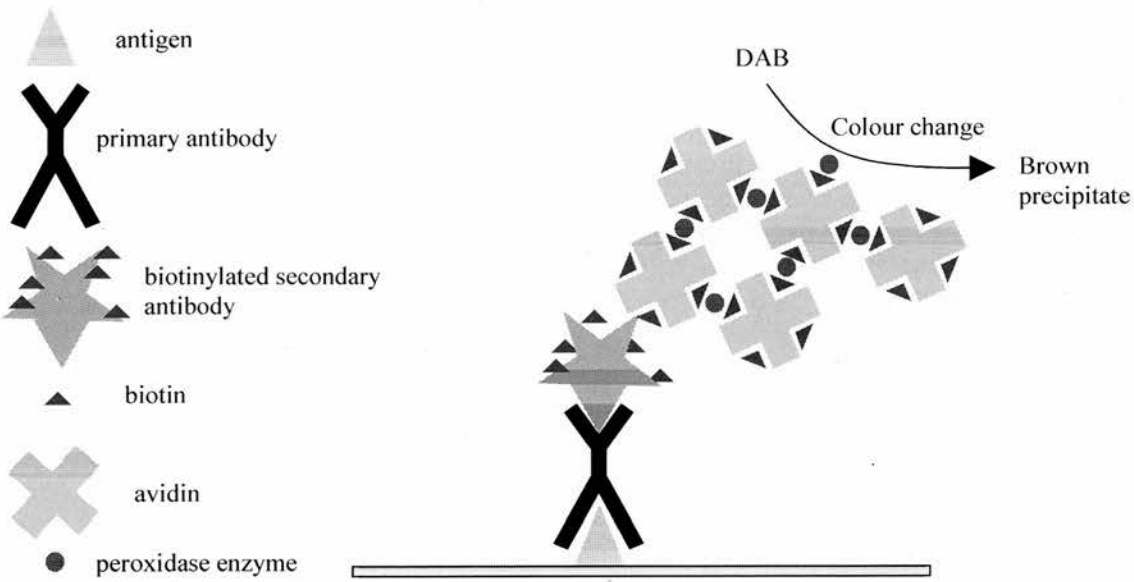


Figure 2.2: Immunohistochemistry using ABC and DAB detection. After blocking, the primary antibody binds to a specific antigen in the tissue/cell. The biotinylated secondary antibody then binds to this. A complex of peroxidase labelled avidin and biotin will then bind to the biotin on the secondary antibody with its' vacant sites. When DAB is added, the peroxidase enzyme causes a reaction which leaves a brown precipitate at the site of the antigen.

2.4.1 Cultured Cell Immunohistochemistry

Cultured cells were fixed for 30 min in 10 % NBF at room temperature then stored in 70 % ethanol until use. Fixed cells were re-hydrated in 50 % EtOH for 2 min then washed in 1 % Triton-X-100 for 10 min. Plates were then washed in PBS (appendix 1.2.1) 3 times for 2 min then endogenous peroxidase activity blocked with 3 % H_2O_2 for 30 min. The cells were washed in PBS twice for 5 min and H_2O for 2 min before adding haematoxylin (a blue non-specific nuclear stain) for 1 min. Scott's solution (appendix 1.2.2), which assists in colour development, was added for 1 min, followed by washing in PBS twice for 2 min. The cells were blocked in 10 % goat serum (appendix 1.2.3) for 20 min then primary antibody added for 2 hours at 4 °C. This was followed by washing in PBS twice for 2 min then the secondary antibody added for 1 hour. After another 2 washes in PBS for 2 min ABC reagent (appendix 1.2.4) was added for 1 hour. Then cells were washed in PBS twice for 2 min then specific staining detected by DAB (appendix 1.2.5) for 4 min before being rinsed and left in tap water.

2.4.2 Immunohistochemistry on Tissue Sections

2.4.2.1 Slide Coating in 3-aminopropylmethoxy-silane (AAS)

Slides were coated with AAS in order for tissue sections to adhere to the slides. A rack of blank slides was dipped in 2 % AAS in acetone 30 times then washed in 3 changes of distilled water, 10 dips each. The slides were then left to dry in an incubator overnight.

2.4.2.2 Block Cutting

Blocks of fixed tissue were placed face down on an ice block for 30 min prior to cutting as paraffin blocks section best when cool and moist. They were cut using a microtome set to a thickness of 5 μm. When a ribbon of sections was cut it was transferred to a water bath at 37 °C to flatten out the sections, which were then separated and mounted on an AAS coated slide. The slides were incubated at 37 °C overnight.

2.4.2.3 Immunohistochemical Procedure for Formalin Fixed Tissue

Slides were de-waxed in histoclear (2 x 5 min) and re-hydrated in graded ethanol (100 % EtOH 20 s, 100 % EtOH 20 s, 95 % EtOH 20 s, 70 % EtOH 20 s, rinse in water). Antigen retrieval was carried out by boiling the slides in 2 l of citrate buffer (appendix 1.2.6) in a pressure cooker for 2 min at full pressure. The slides were then blocked in 3 % H_2O_2 for 20 min to block endogenous peroxidase activity, then rinsed in tap water and washed in TBS (appendix 1.2.7)(2 x 5 min). The sections were then drawn around with a wax pen (to contain fluid) and blocked in goat serum (appendix 1.2.3) for 30 min. The slides were then drained and excess block wiped off, before adding the primary antibody (100 µl per section) for an overnight incubation at 4 °C. The slides were then washed in TBS (2 x 5 min) and wiped then the secondary antibody added (100 µl per section) for 30 min. Slides were again washed in TBS (2 x 5 min) and wiped before adding ABC (appendix 1.2.4) for 30 min. Slides were washed in TBS (2 x 5 min) and wiped. The positive staining was then detected by application of the peroxidase substrate, DAB (appendix 1.2.5), which produces a brown stain. Colour development was monitored microscopically and stopped by washing in H_2O. Cells were lightly counterstained in haematoxylin for approximately 8 min, washed in H_2O then dipped very briefly in acid-alcohol and rinsed immediately in H_2O. The slides were then dipped in Scott's solution (appendix 1.2.2) to develop the blue colour and washed in H_2O before being dehydrated in graded ethanol (70 % EtOH 20 s, 95 % EtOH 20 s, 100 % EtOH 20 s, 100 % EtOH 20 s, histoclear 5 min, xylene 5 min) then mounted with coverslips using Pertex.

2.4.2.4 Haematoxylin and Eosin stain (H and E)

Tissue sections were de-waxed and re-hydrated as above then rinsed in tap water prior to haematoxylin staining for 5 min. Then slides were rinsed briefly in acid-alcohol, Scott's solution then Eosin for 20 s with a tap water rinse between each stage. Sections were then dehydrated as above and Pertex mounted.

2.5 Messenger RNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Polymerase chain reaction (PCR) was the first practical system for *in vitro* amplification of DNA, invented by Kary Mullis in 1983 (Mullis et al 1986, Shampo & Kyle 2002). Two synthetic oligonucleotide primers, which are complementary to two regions of the target DNA to be amplified (one for each strand), are added to the target DNA in the presence of excess nucleotides and Taq polymerase, a heat stable DNA polymerase. In a series of cycles, the target DNA is repeatedly denatured (around 90 °C), annealed to the primers (around 60 °C) and a new strand extended from the primers (72 °C). The new strands then act as additional templates in the next cycle, thus amplifying the DNA exponentially (*Figure 2.3).*

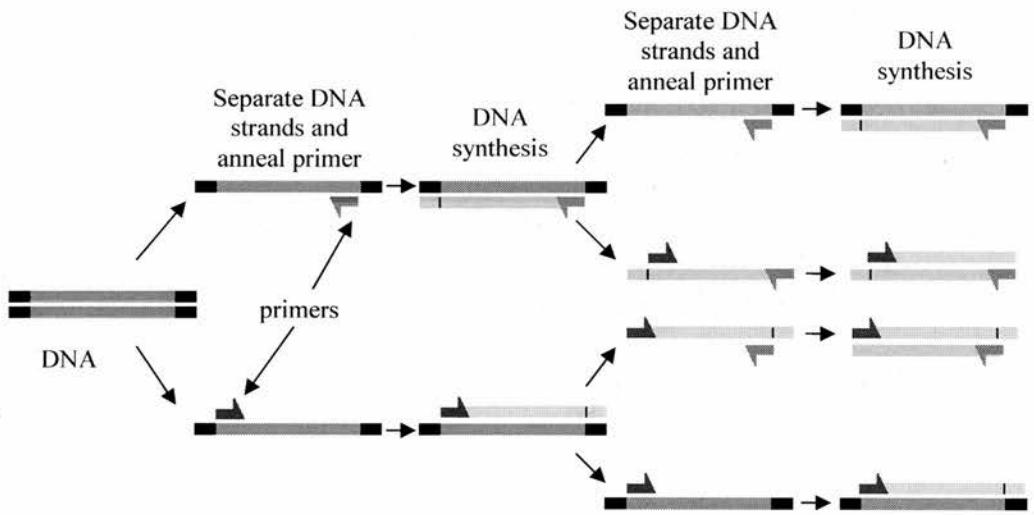


Figure 2.3: DNA amplification by PCR. Each cycle consists of DNA separation, primer annealing and DNA synthesis.

2.5.1 Messenger RNA extraction

For cell extraction, medium was pipetted off the cultured cells, which were then washed in DPBS. Cells from 4 wells (identically treated) of a 24 well culture plate were combined in 1 ml tri-reagent, a guanidine thiocyanate based reagent (Chomczynski & Sacchi 1987). This was passed several times through a pipette to form a homogenous lysate before being transferred to 1.5 ml nuclease-free tubes and kept on ice for the remainder of the procedure. Tissue samples were either collected directly into tri-reagent (1 ml for 50-100 mg tissue) or cultured samples were washed twice in DPBS then added into tri-reagent. This was homogenised for 1 min to break up the tissue. To each 1 ml of tri-reagent from tissue or cells, 200 µl of chloroform was added and the tubes shaken vigorously for 15 seconds, then left to stand for 10 minutes before being centrifuged in a biofuge at 4 °C, 10,000 g for 20 min. This removed the pink organic phase containing protein and a white layer of DNA that became trapped beneath the gel layer. The top colourless, aqueous phase, containing RNA was pipetted into 1.5 ml eppendorf tubes. To each sample, 500 µl of isopropanol was added and tubes were inverted several times then left on ice for 1 hr. Samples were then centrifuged at 4°C, 10, 000 g for 15 minutes to precipitate out an RNA pellet. The isopropanol was pipetted off and 70 % ethanol (prepared with nuclease-free water) added to each eppendorf. After a brief, gentle vortex, the samples were centrifuged at 4°C, 10,000 g for 10 min. The ethanol was removed from each tube, then the tubes left open for 10 min to dry. The pellets were then suspended in 10 µl nuclease-free water.

The concentration of total RNA in the sample was then determined by spectroscopy using GeneQuant Pro. This machine automatically calculates the concentration (A_{260}) and quality ($A_{280/260}$) of RNA. If RNA is free of DNA the ($A_{280/260}$) ratio should be ≥ 1.7. All samples were then diluted using nuclease-free water to a concentration of 1 µg/µl. If the RNA concentration was too low, the volume of sample required to give 1 µg RNA was calculated.

2.5.2 Deoxyribonuclease Treatment

To remove contaminating genomic DNA from the extraction process, messenger RNA samples were DNAse treated in a 10 µl reaction consisting of 1 µl mRNA (of 1 µg/µl stock), 1 µl deoxyribonuclease (1 U/µl stock), 0.1 µl RNAse inhibitor (20 U/µl stock), 1 µl 10x buffer, 6.9 µl nuclease-free water. This reaction mixture was incubated at 37 °C for 30 min, 1 µl stop solution added then incubated for a further 10 min at 70 °C. DNAse treated RNA was then stored at -70 °C.

2.5.3 Reverse Transcription of RNA

In order to obtain cDNA to assess specific genes of interest, DNAse treated mRNA samples were reverse transcribed in a 20 µl reaction mix consisting of 2 µl mRNA (0.1 µg/µl DNase-treated stock), 2 µl dNTP's (10 mM stock), 1 µl RNAse inhibitor (40 U/µl stock), 1 µl reverse transcriptase (50 U/µl stock), 1 µl random hexamers (5 µM stock), 2.4 µl $MgCl_2$ (25 mM stock), 2 µl 10x buffer and 8.6 µl nuclease-free water. Samples were incubated at room temperature for 10 min, 42 °C for 60 min then 95 °C for 10 min. The cDNA was then stored at – 20 °C. A sample with nuclease-free water in place of mRNA was prepared to use as a negative control for reverse transcription (RT).

2.5.4 RT-PCR

cDNA samples were tested for a house keeping gene, glucose-6 phosphate dehydrogenase (G-6-PDH) to check the extraction and RT stage had been successful. This is a suitable house-keeping gene as it is constitutively expressed and does not vary with the added treatments. The RT negative control was included in the PCR, in the case of any contamination, to aid the isolation of the source. A PCR negative was also included in each run, with nuclease-free water in place of cDNA. Amplified PCR products were run on a 1.5 % agarose gel (appendix 2.4/section 2.7.2.6) containing ethidium bromide and visualised and photographed under UV light. A 100 bp DNA ladder was also run to show product size. If no contamination was apparent, cDNA samples were then amplified for the gene of interest. Primers were designed to amplify a

region of the gene. Each 10 µl PCR mix consisted of the following: 1 µl 10x buffer, 0.6 µl $MgCl_2$ (25 mM stock), 0.2 µl dNTPs (10 mM stock), 1 µl each of forward and reverse primers (5 µM stock), 4.7 µL nuclease-free water, 0.5 µl Taq DNA polymerase (5U/µl) and 1 µl cDNA. The samples were optimised for annealing temperature using a gradient PCR machine, and also $MgCl_2$ concentration (the above shows 1.5 mM $MgCl_2$, this can be adjusted along with nuclease-free water to change the concentration). The samples were run on a programme as follows:

Stage 1:	95 °C	5 min
Stage 2:	95 °C	30 s
(35 cycles)	62 °C	30 s
	72 °C	1 min 30 s
Stage 3:	72 °C	10 min

2.5.5 Quantitative PCR using the Lightcycler

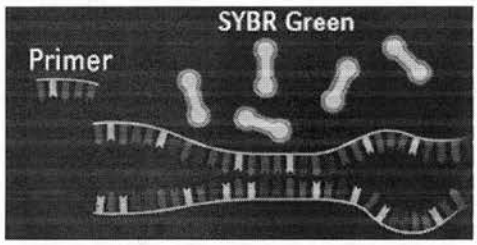
The Lightcycler (Roche) was used to amplify and quantify cDNA in a cyclic process of DNA denaturation, specific primer annealing and primer extension to exponentially increase the primer specified DNA fragment. cDNA sequences for target genes were obtained from Genebank and primers were designed against a segment of sequence that displayed low homology with other genes of the same family and where possible, spanning an intron. This allowed identification of genomic contamination. If genomic DNA remains in the sample even after DNAse treatment, 2 peaks with different melt curves would be produced when using melt curve analysis, or 2 bands would be visible on an agarose gel.

Real time quantification of this process allows cDNA synthesised after each PCR step to be visualised by fluorescence of SYBR Green 1. This is a DNA binding dye, which by binding to the minor groove of double stranded DNA, greatly enhances its fluorescence. During the stages of PCR, different intensities of fluorescence are detected depending on the amount of double stranded DNA present. After denaturation, all DNA present is

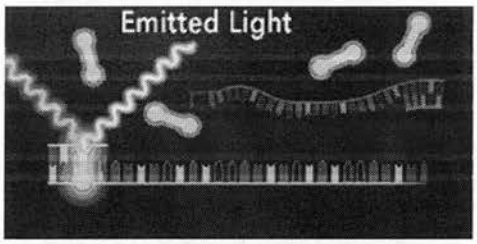
single-stranded, hence no SYBR Green 1 is bound giving a low signal. During annealing, primers hybridise to the target sequence resulting in small amounts of SYBR Green 1 binding and a low fluorescence. During elongation as the primers are extended, more SYBR Green 1 becomes bound hence an increase in the signal. At the end of elongation, all DNA is double stranded and the maximum amount of dye is bound. The fluorescence (530 nm) is recorded at this stage and increasing amounts of PCR product can be monitored from cycle to cycle (*Figure 2.4*).

The quantification is based on calculations taking into account a housekeeping gene and the gene of interest. Choice of the house-keeping gene is critical. It must be quantifiable over the same range as the gene of interest and be amplified with the similar efficiency. It must be constitutively expressed, being equal in all samples and not variable with treatment, and optimally synthesised at the same primer annealing temperature as the gene of interest, and with the same $MgCl_2$ concentration. The primer annealing temperature was optimised using a gradient PCR machine, and the $MgCl_2$ concentration optimised on the Lightcycler. $MgCl_2$ is present in the SYBR Green1 master mix used for reactions at a concentration of 1 mM, so additional $MgCl_2$ can be added to optimise.

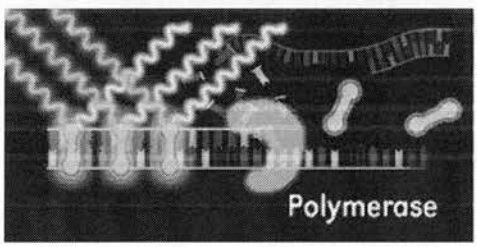
Each 10 µl reaction mix contains the following: 1 µl SYBR Green1 mastermix, 5.4 µl nuclease free water, 0.5 µl each forward and reverse primers, 1.6 µl $MgCl_2$ (of 25 mM stock – this volume and water volume vary according to optimum $MgCl_2$ concentration), and 1 µl cDNA. The samples are run on the Lightcycler using a programme set up as shown:



At the start, the reaction mixture consists of denatured DNA, primers, and dye. The unbound dye fluoresces weakly, producing a small background signal which is subtracted during computer analysis



After annealing of the primers, some dye molecules can bind to the double strand. This increases the fluorescence of the sample.



During elongation, more and more dye molecules bind to the newly synthesised DNA. Monitoring the reaction continuously thus shows real time increases in fluorescence. Upon denaturation of the DNA for the next heating cycle, the dye molecules are released and the fluorescence signal falls.

Figure 2.4: Diagrams to show the binding of SYBR Green dye throughout the stages of PCR using the Lightcycler. Diagrams from www.lightcycler-online.com.

Programme:

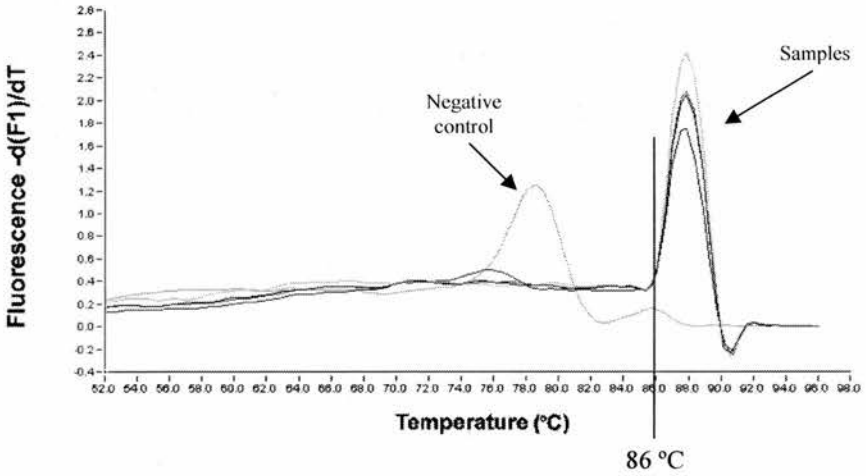
Stage 1:	95 °C	10 min
Stage 2:	95 °C	15 s
(45 cycles)	61 °C	5 s (annealing temperature is optimised for gene)
	72 °C	15 s
	87 °C	1 reading (measurement temperature is optimised for gene)
Stage 3:	95 °C	0
	50 °C	15 s
	50 -99 °C	0.1 °C/s
Cool:	40 °C	30 s

In order to identify a temperature to measure the fluorescence of specific product only, avoiding non-specific double stranded DNA, for example primer dimers, melt curve data was analysed (*Figure 2.5a*). The samples are cooled then gradually reheated with constant fluorescence readings. The graph of fluorescence against temperature shows a sharp drop in fluorescence when the specific DNA is denatured. There is also, at a lower temperature, another drop in fluorescence as primer dimer pairs (if present) are denatured. The temperature selected is between these 2 temperatures, so primer dimers have denatured, but the DNA of interest will remain double stranded.

Relative quantification of specific DNA involves normalisation to a known relative concentration of serially diluted standards. This should be reverse transcribed at the same time as samples to reduce reverse transcription variation. The samples should be in the same range as the standard curve, in which a graph of the cycle number versus the log of concentration should have a gradient of approximately -3.3 (*Figure 2.5b*). To reduce reaction variation, a master mix of reagents should be prepared and samples run in duplicate. In each run on the Lightcycler, which is capable of holding 32 capillaries, 5 controls for the gene of interest and 5 controls for a house keeping gene are included. This leaves space to run 5 samples in duplicate for the gene of interest and the housekeeping gene. Results are given as a ratio of the gene of interest to the

housekeeping gene to normalise the samples to one another allowing for direct comparison.

(a)



(b)

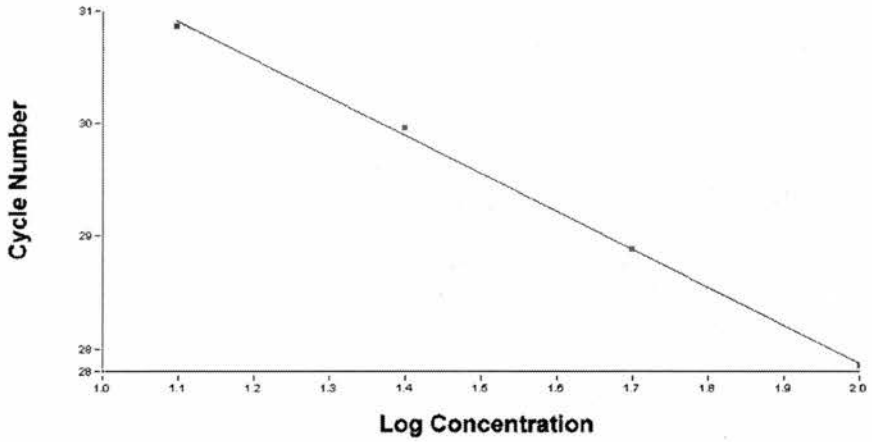


Figure 2.5: Graphs from Lightcycler. (a) Melt curve analysis. A primer dimer can be seen in the negative control. It can be seen that the best temperature to take a measurement in this case is 86 °C. (b) A standard curve is produced for each gene using control standards. An ideal slope for this curve is -3.3. Here, the slope is -3.361. From this, the concentrations of all unknown samples can be calculated.

2.6 Assay Techniques

2.6.1 Protein Assay

This protocol has been adapted from the original Lowry assay method (Lowry et al 1951). Standards were prepared using BSA diluted in distilled water. The standards ranged from 5mg/ml to 0.078 mg/ml and 20 µl of each standard was used to give a working range of 100 µg to 1.56 µg protein. 20 µl of each standard or sample (diluted in water as required) was added to a 96 well plate followed by 100 µl solution A (appendix 1.4.1.5) to all wells. This was mixed on a plate shaker at room temperature for 10 min before adding 10 µl Folin and Ciocalteu's Reagent (appendix 1.4.1.6) and mixed on the plate shaker for a further 30 min before being read at 650 nm using the Softmax programme. The Softmax programme will draw a standard curve according to densitometric readings of the standards then calculate values for unknown samples using this standard curve. A sample protein assay standard curve is shown (*Figure 2.6a*).

2.6.2 PGE_2 Assay – Competitive ELISA

The PGE_2 assay is an example of a competitive enzyme-linked immunosorbent assay (ELISA). A competitive ELISA makes it possible to obtain an estimate of the amount of a particular antibody or antigen, even when these cannot be isolated from the medium in which they are found. Here, a plate is coated with purified donkey anti-rabbit IgG and then blocked to remove non-specific binding (*Figure 2.7*). The sample containing the substance to be measured is then added with anti-sera and a link (biotinylated PGE_2). The anti-serum, rabbit anti-PGE_2, will bind to epitopes coating the wells. The anti-serum can also recognise identical epitopes in the sample and in the link. If the sample being measured contains a high concentration of the PGE_2, then the majority of the anti-serum will have become conjugated to it. However, if there is a low concentration, the anti-sera will have bound to the link. The conjugate, streptavidin peroxidase, is then added which binds to biotin in the link. Excess strepatavidin peroxidase is washed off. When the enzyme substrate, TMB is added, streptavidin peroxidase catalyses a reaction which

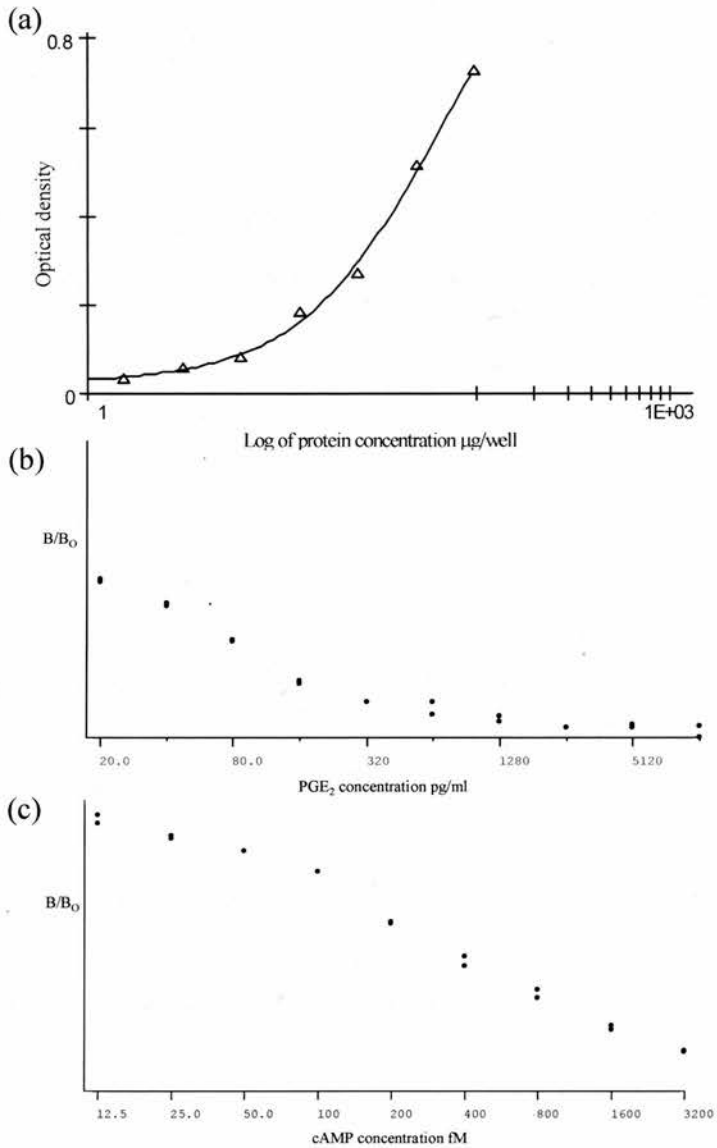


Figure 2.6: (a) Standard curve for protein assay by Softmax. This curve fit is 4-parameter with the formula: y = (A-D)/(1+(x/C)^B)+D. (b) and (c) Standard curves for PGE_2 and cAMP assays using Assay Zap. The Y axis is a ratio of B (optical density – NSB) over B_o.

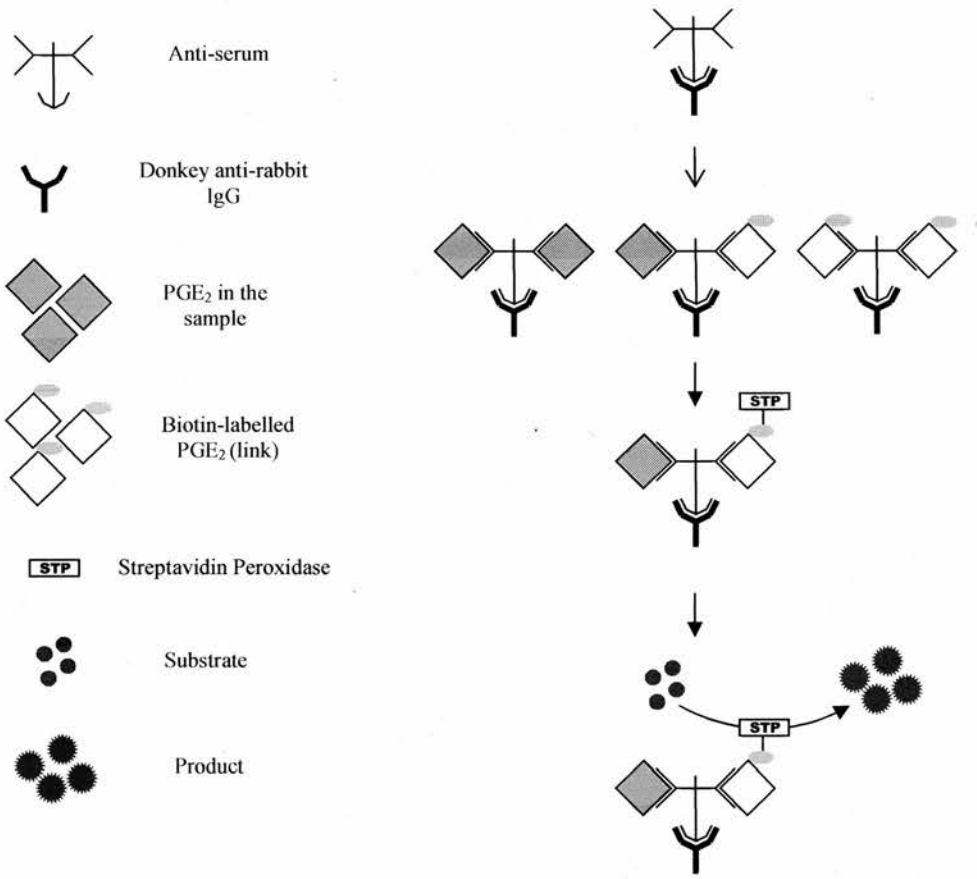


Figure 2.7: PGE_2 ELISA, an example of a competition ELISA reaction. Anti-serum binds to donkey anti-rabbit epitopes in the wells. Then PGE_2 in the sample competes with the biotin-labelled link for anti-serum binding sites. Streptavidin peroxidase then binds to the biotin on the bound link and converts the substrate into a coloured product which can be measured. The greater the level of PGE_2, the lower the concentration of coloured product.

converts the substrate into a coloured product, which can be measured. The amount of the substance being measured in the original samples can be calculated by comparing values obtained by using a standard curve.

This method has been adapted from one previously described (Kelly et al 1989). On collection, samples were treated 1:1 with methyloximating solution (MOX) (appendix 1.4.2.1) overnight at 4 °C to stabilise PGE_2 and prevent conversion to the PGA form. This treatment causes chemical derivitisation of the prostaglandin via the ketone link, forming a methoxime at C9 in place of the oxo group. 96-well plates were coated with purified donkey anti-rabbit (DAR) serum using the direct binding procedure. Briefly, 100 µl purified DAR serum diluted 1:200 with PBS was added to each well and sealed and left over night at 4 °C. The contents were then flicked out and 300 µl/well Dry Coat solution (appendix 1.4.2.2) was added and incubated for 1 hr at room temperature. The contents were flicked out and wells washed 4 times in wash buffer (appendix 1.4.2.3) before use.

Standards were prepared at a range from 10240 – 20 pg/ml using 25% MOX buffer (appendix 1.4.2.5). 100 µl standard was added per well. 50 µl samples + 50 µl buffer containing Tween (appendix 1.424) and no MOX, bringing the MOX concentration to 25 % as the standards. Additionally in the wells, there was 50 µl antiserum (diluted in ELISA buffer + Tween to 1 in 50,000) + 50 µl link (diluted 1 in 3 million with phosphate buffer, appendix 1.4.2.6). Wells were also prepared containing only buffer + 25% MOX with (B_o) and without (NSB) antiserum. Quality control wells were also set up with a standard concentration of 200 pg/ml. The link increases the sensitivity of the assay via proline coupling to a PG, making it more antigenic. This enables the free PG in the sample to displace the biotin-labelled link for antiserum binding sites. The above is left overnight at 4 degrees and is followed by washing 4 times in wash buffer, including once for 30 s on a plate shaker. Streptavidin was diluted in buffer with no tween to a concentration of 0.125 U/ml and 100 µl pipetted into all wells. This was

shaken for 25 min at room temperature then washed 4 times as previously. TMB substrate (appendix 1.4.2.7) is then added at 200 µl per well. Plates were then left for approximately 10 minutes before being quenched with 50 µl 2N sulphuric acid and read on a plate reader at 450 nm within 30 min of quenching. Results were analysed and quantified using Assay Zap and Microsoft Excel. Assay Zap is a programme which will use an optical density measurement to draw a standard curve for the standards, and then calculates a value using this curve for the unknowns. A sample standard curve for a PGE_2 assay is shown (*Figure 2.6b*).

2.6.3 Cyclic AMP Assay

The cAMP assay was carried out using an enzyme-immunoassay (EIA) system kit. This assay is based on the ELISA method described above. However, instead of a labeled link being added, peroxidase labeled cAMP was added to compete for anti-sera binding with the cAMP in the samples. Standards were prepared by serial dilution to a range of 12.5 - 3200 fM by diluting a stock standard (3200 fM) in lysis reagent 1B. The kit comes with a 96-well plate coated with donkey anti-rabbit IgG. This was washed 4 times with wash buffer prior to use. Into each well, the following was added: 100 µl lysis reagent 1B (NSB and 0 standard wells)/100 µl standard/100 µl sample and 100 µl lysis reagent 2B (NSB only)/100 µl antiserum. This was covered and left at 4 °C for 2 hours. Then 50 µl cAMP-peroxidase conjugate was added into all wells and the plate covered and incubated at 4 °C for 60 min. The wells were then aspirated and washed 4 times with wash buffer. Then 150 µl enzyme substrate was dispensed into each well and mixed on a plate shaker at room temperature for 60 min. Following this, the reaction was quenched by addition of 100 µl 1 M sulphuric acid and the optical density determined at 450 nm within 30 min. Results were analysed and quantified using Assay Zap and Microsoft Excel. A sample standard curve for a cAMP assay is shown in *Figure 2.6c*.

2.7 Electrophoresis Based techniques

2.7.1 Sample Preparation for Electrophoresis

2.7.1.1 Freeze Drying

A known volume of culture medium was collected in a 2 ml Sarstedt tube and covered with parafilm with small holes pierced in the top and frozen at –70 °C before use. The samples were placed in the freeze drier and vacuum pump apparatus on a suitable rack and freeze-dried at a pressure of 6-8 mbar until dry (about 6 hr for 1 ml). When dry, the pressure was released and samples stored capped at –20 °C until reconstituting in a known volume of distilled water.

2.7.1.2 Dialysis

Some samples require a dialysis step to "clean" them up for electrophoresis. The dialysis tubing used here was capable of retaining proteins 12 kB or larger. This also allows removal of salts in the culture medium and salts associated with the folded protein, which stops them interfering with the complex separation procedure of electrophoresis. Tubing was cut to the required length (10 cm for 1 ml sample) and added to very hot water for 5 min prior to use to render it pliable. A clip was placed on one end of the tubing and the other end opened and the sample pipetted in. The tubing was then sealed with another clip. Samples were immersed in distilled water and dialysed overnight at 5 °C stirring very slowly on a magnetic stirrer. When completed, the samples were retrieved and stored at –20 °C and freeze-dried as appropriate.

2.7.1.3 Homogenisation of Cultured Tissue Samples

Samples collected in lysis buffer (appendix 1.511/2) were frozen at –20 °C or -70 °C until homogenisation. Using the Polytron PT 3100 with the 7 mm probe, the sample was homogenised for 2 x 30 sec on ice at the highest setting. The probe was cleaned with DPBS between samples. The samples were then centrifuged at 10,000 g for 20 min at 4 °C. The supernatant was collected and stored at – 20 °C before use (samples used for the cAMP assay were stored at –70 °C for 10 days maximum before use). The samples

used to measure ERK phosphorylation were sonicated for 10 min prior to the centrifugation step. This breaks up nucleotides in the sample making it less 'gooey' and easier to use.

2.7.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.7.2.1 Principles of SDS-PAGE

SDS-PAGE is a separation technique commonly used to separate proteins by molecular weight. The relative mobility of a protein in an SDS-PAGE gel is related to its molecular weight. A standard curve is constructed with proteins of known molecular weight by plotting the logarithms of their molecular weights against the relative mobilities of the proteins. The relative mobility of an unknown protein is then fitted to the curve to determine its molecular weight. Polyacrylamide gels are formed by co-polymerisation of acrylamide and bis-acrylamide, which is initiated by TEMED and ammonium persulphate. SDS is added to dissociate proteins into their individual polypeptide subunits and gives a uniform net charge along each denatured polypeptide. The percentage of acrylamide in the gel can be varied to visualise a range of protein molecular weights, as proteins migrate faster through a lower percentage gel. Ammonium persulphate is very hygroscopic so the gel is degassed prior to pouring and the ammonium persulphate solution is prepared freshly before making a gel.

2.7.2.2 General Electrophoresis Method

The discontinuous system used here (*Figure 2.8*) involves a resolving gel with a stacking gel on-top in which the proteins are loaded. Using the Biorad minigel system, resolving gel (see appendix 2) was prepared and left to set topped with sec-Butanol to create a level top on the gel. This was then washed off and the gel left overnight to equilibrate, topped with stacking gel buffer (diluted 1 in 5 with water). This buffer was washed off and stacking gel was then poured on-top with the chosen spacers to form wells. This was left for 1.5 hr to set. The gel apparatus was then filled with running tank buffer and the samples loaded into wells. Molecular markers were always also loaded. Broad range markers of roughly 30 – 210 kDa were used on 7.5 % gels, and low range

markers of around 20 – 120 kDa were used for 10 and 12 % gels. Gels were run at a constant voltage of 100 V for an appropriate length of time.

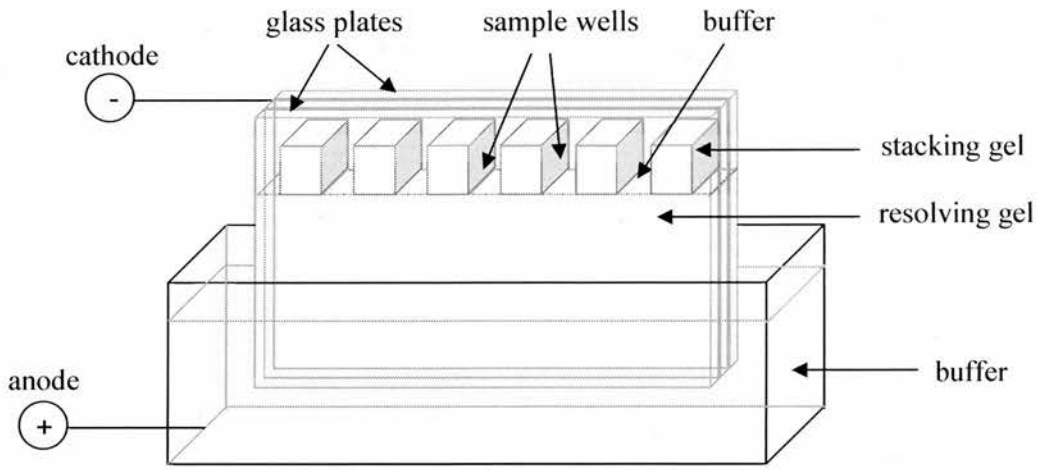


Figure 2.8: Assembly of gel apparatus for the discontinuous gel electrophoresis system. The resolving gel is prepared and left to equilibrate overnight before being over-lain with stacking gel using combs to create wells where the sample is loaded before being electrophoresed.

2.7.2.3 Western Blotting

Western blotting detects the presence of proteins in a sample. Samples are separated by SDS-PAGE and then the proteins on the gel are transferred by electrophoresis onto a thin sheet such as nitro-cellulose membrane (*Figure 2.9*). The proteins bind to this membrane and are immobilized. These proteins are ready accessible to detection using specific labeled antibodies.

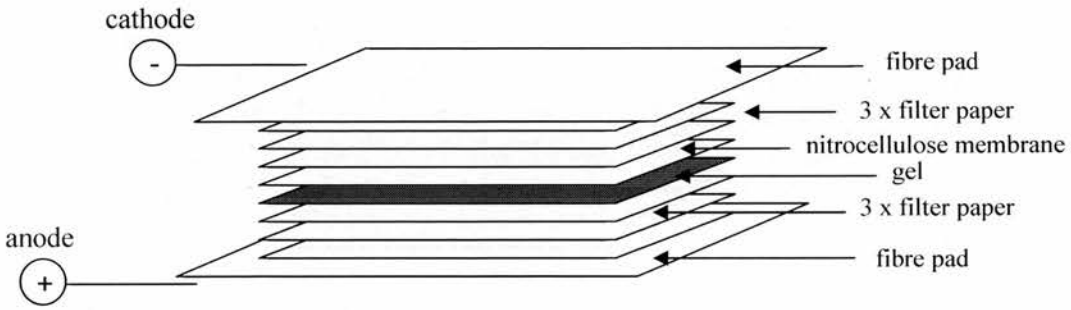


Figure 2.9: Assembly of a "gel sandwich" for Western blot transfer. The layers are kept moist with transfer buffer at all times and rolled with an autoclaved glass pipette to remove all bubbles and to ensure layers are in contact in all places.

The method used for Western Blotting has been adapted from the original method by Towbins et al. (Towbin et al 1979). Gels were prepared (appendix 2.1) and loaded with samples mixed 1:1 with sample application buffer (appendix 1.5.1.3). They were electrophoresed in running tank buffer (appendix 1.5.1.4) for 2 hours at 100 V. Following this, gels were equilibrated in transfer buffer (appendix 1.5.1.5) for 30 min. Nitro-cellulose membranes and filter paper were also steeped in transfer buffer for 30 min and 10 min respectively, prior to use. The "gel sandwich" was constructed and electrophoresed with a Bio-Rad Ice Cooling unit at 100 V for 60 min. Gels could then be stained using Coomassie Blue (appendix 1.5.2.9) for 60 min to check protein transfer. Membranes were then blocked in 5 % BSA (appendix 1.5.1.8) in TTBS solution (appendix 1.5.1.7) overnight at 5 °C, prior to adding the primary antibody for 2.5 hours. Membranes were then washed in TTBS (1x 15 min, 2x 5 min) then a horse radish peroxidase (HRP) linked secondary antibody was added for 1 hour. The membranes were then washed again (1x 15 min, 4x 5 min) and then the proteins were detected using

enhanced chemiluminescence (ECL). This method is based on the luminescent properties of luminol. In the presence of H_2O_2, HRP on the nitrocellulose membrane catalyses the oxidation of cyclic diacylhydrazides, such as luminol. Oxidation causes luminol to become excited. It then slowly decays to the ground state, with a half life of 60 min, by emitting light. This light is detected by exposing the membranes to autoradiography film. A strong enhancement of the light emission is achieved by enhancers, such as phenolic compounds (Thorpe & Kricka 1986, Thorpe et al 1985, Whitehead et al 1979). After exposure, membranes were developed in a processor or by hand. Membranes were then stripped for 45 min using stripping solution (appendix 1.5.1.9), washed (2x 5 min) in TTBS and then either stored at 5 °C for future use, or re-blocked and probed.

2.7.2.4 Gelatin Zymography

Gelatin zymography detects the activity of latent and active forms of the gelatinases, MMP-2 and MMP-9 using a method previously described (Rawdanowicz et al 1994). The resolving gel contains gelatin which is a substrate for the gelatinases. The proteins are visualised as a site of enzymatic activity, identified by a white band when the gel is stained blue. The latent forms of the gelatinases are also seen as SDS present in the gel activates them via a conformational change to expose the active site, as well as dissociating active forms from their inhibitors. A sample zymogram is shown in *Figure 2.10(a)*. A dimer of the latent form of MMP-9, and a complex of MMP-9 and lipocalin are also visualised on the zymograms (Kolkenbrock et al 1996).

Gels were prepared (appendix 2.2) and samples mixed 1:1 with sample application buffer (appendix 1.5.2.1) loaded into the wells. A sample of characterised amniotic fluid, containing active and latent forms of both MMP-2 and MMP-9 was also run as a control. Gels were electrophoresed in running tank buffer (appendix 1.5.2.2) at 100V for approximately 90 min. Gels were washed twice with Triton-X wash buffer (appendix 1.5.2.5), twice using TBS wash buffer (appendix 1.5.2.3), then incubated in zymography digestion buffer (appendix 1.5.2.6) for 18 hours at 37 °C. Gels were then washed twice

with TBS wash buffer, immersed in staining solution (appendix 1.5.2.9) for 3 hours at room temperature, then de-stained using de-staining solution (appendix 1.5.2.8) to reveal discrete bands where gelatin has been hydrolysed by gelatinase activity. The zymograms were then scanned and semi-quantified by densitometry using Biorad Quantity One software.

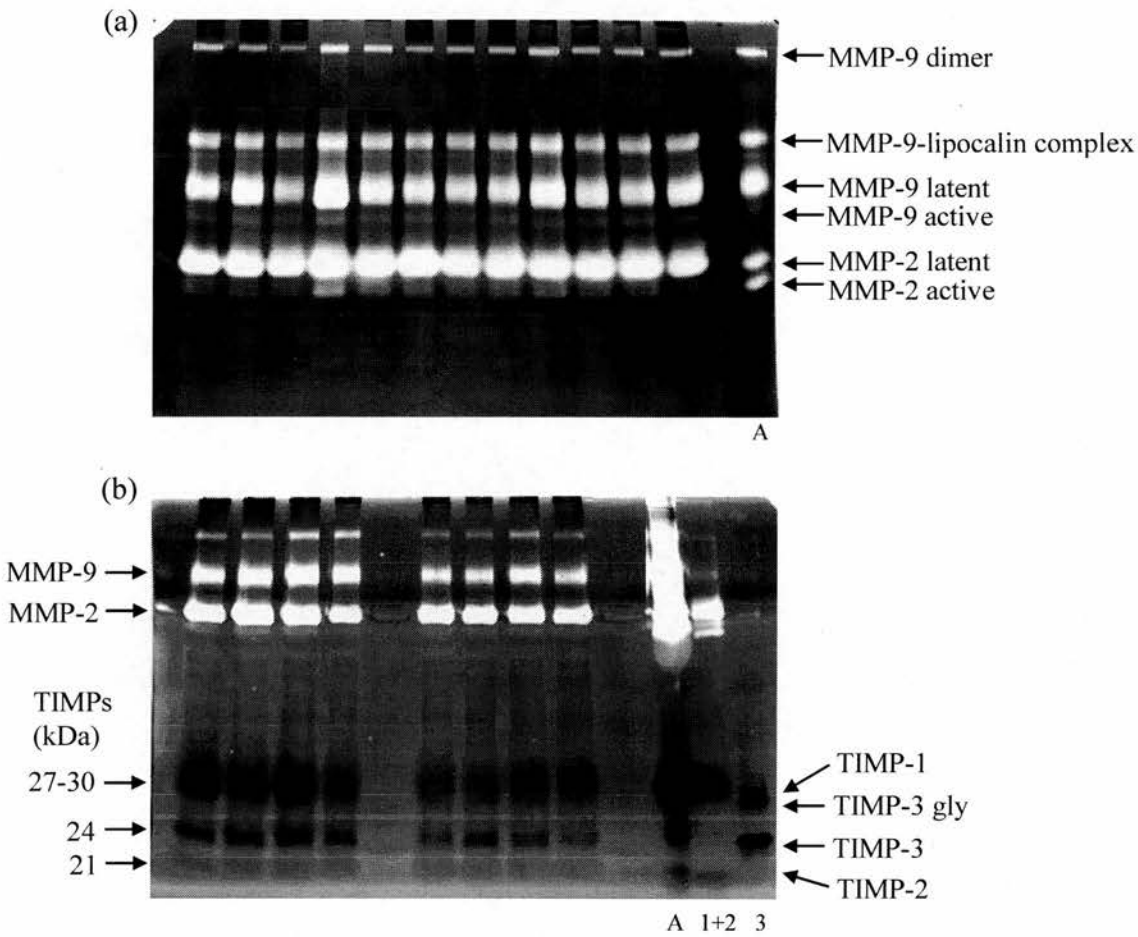


Figure 2.10: Example of (a) a zymogram and (b) a reverse zymogram. A= amniotic fluid control, 1+2 = TIMP-1 and -2 standard, 3 = TIMP-3 standard.

2.7.2.5 Reverse Zymography

Reverse zymography detects activity of TIMPs. Samples were separated by SDS-PAGE, using gels containing gelatin and an MMP preparation (from BHK-21 cells that constitutively express proMMP-2; University Technologies Inc). This method has previously been described (Hampton et al 1995).

Gels were prepared (appendix 2.3) and loaded with samples mixed 1:1 with sample application buffer (appendix 1.5.2.1), then run for approximately 90 min at 100V. The gels were washed in Triton-X wash buffer (appendix 1.5.2.4) then incubated in reverse zymography digestion buffer (appendix 1.5.2.7) for 17 hours at 37 °C. The gels were stained (as for zymography) for 3 hours using staining solution then de-stained with de-staining solution. The presence of TIMPs was detected by their discrete inhibition of MMP activity, seen as a dark band on a lighter background (*Figure 2.10b*). TIMPs were identified and characterised by comparison with molecular weight markers, with control standards of conditioned medium containing mouse TIMP-1, -2 and –3 expressed by transfected BHK cells and recombinant TIMP-2. This human TIMP-2 standard shows different mobility to the mouse form (observed at 21 kDa by reverse zymography under non-reducing conditions and 26 kDa by Western Blot under reducing conditions)

2.7.2.6 Agarose Gels

Agarose gels are commonly used to separate nucleic acids. Agarose is a neutral, linear polysaccharide in agar, consisting of repeating galactose and 3,6-anhydrogalactose. Powdered agarose was mixed with electrophoresis buffer (appendix 2.4) and boiled then poured into a gel tank with a suitable comb to create wells and allowed to cool. Ethidium bromide was added to the hot solution (in a fume hood) as this binds to nucleic acids and fluoresces under UV light allowing visualisation of the genetic material. Samples which had undergone PCR were mixed with a loading dye and loaded into wells in the gel along with a DNA ladder (consisting of 11 double-stranded DNA fragments with sizes 100-1500 bp to serve as a reference indicator). The gels were electrophoresed for 1 hr in

TAE buffer (appendix 1.3) at 100V and then examined and photographed under UV light using a transilluminator.

2.8 Statistical Analysis

All statistical analysis was carried out using Microsoft Excel. The significance values were calculated using a paired student T-test. Significance was indicated when $p < 0.05$ or $p < 0.01$.

Chapter 3: Prostaglandin Receptors in Human Uterine and Gestational Tissue at Parturition

3.1 Introduction and Aims

Prostaglandins have many roles within the human uterus during pregnancy and parturition. These roles are likely to be mediated via specific prostaglandin receptors (see section 1.3). The receptors, EP1, 2, 3, 4, DP, FP and IP have all been identified in the human pregnant myometrium (Duckworth et al 2002, Erkinheimo et al 2000, Leonhardt et al 2003, Matsumoto et al 1997b, Senior et al 1993). There is not a lot of published evidence of prostaglandin receptors in any other tissues involved in pregnancy besides the myometrium in the human. FP has been detected in human amnion-decidua complexes (Fukai et al 1984) and also in the human placenta (Vielhauer et al 2004).

In the myometrium, the prostaglandin receptors can be split into 2 groups – relaxant, and contractile, by their effect on smooth muscle cells. DP, EP2, EP4 and IP are relaxant and act by increasing the intracellular cAMP concentration, and EP1, EP3, FP and TP can be considered contractile and act by either decreasing cAMP concentrations or increasing intracellular Ca^{2+} concentrations (Coleman et al 1994). The interaction between the release of prostaglandins and their specific receptors might therefore participate in maintenance of uterine quiescence during gestation, and uterine contractions during labour at term and preterm. Comparing the receptor expression and distribution levels between non-labouring and labouring myometrial samples could indicate if this plays a role in uterine quiescence and contractility.

Prostaglandins have also been implicated in regulation of MMP expression and activation in human fetal membranes and decidua (McLaren et al 2000b, Ulug et al 2001), thus affecting extracellular membrane degradation which is involved in rupture of the fetal membranes at term and preterm. This regulation is likely to occur via prostaglandin receptors. To establish the location of the receptors within these tissues would test this hypothesis. A similar mechanism is likely to explain placental detachment from maternal tissues at labour. Collagenase activity in the placenta has

been found to increase during labour (Rajabi et al 1990), particularly MMP-9 (Xu et al 2002) and prostaglandins may be involved in inducing this.

Nuclear prostaglandin receptors have also been identified, though as yet there is no evidence for any in gestational tissues. However, prostaglandin signalling may still occur in the nucleus without these receptors. Other targets for the actions of prostaglandins within the nucleus are peroxisome proliferator activated receptors (PPARs), of which several isoforms have been identified in gestational tissue and JEG3 cells (Helliwell et al 2004, Keelan et al 1999b).

The control by which prostaglandins exert their effects via their receptors depends on an intricate balance of receptor and ligand expression. It follows that regulation of a combination of prostaglandin receptor levels and expression, and enzymes involved in prostaglandin metabolism all contribute to balance the levels to which prostaglandins exert their specific effects during labour. The aim of this chapter is to detect and localise PG receptors in human uterine and gestational tissue at term and preterm, using samples from patients in labour and not in labour. The receptors that are focused on in this chapter are EP2, EP4 and FP. PGDH will also be immunolocalised to compare the sites of prostaglandin metabolism to their sites of action.

3.2 Methods

3.2.1 Identification of PG Receptor mRNA by PCR

For this experiment, n=6 for each of amnion, chorio-decidua, placenta and JEG3 samples. RNA was extracted according to section 2.5.1-2.5.3. The tissue samples were collected directly into tri-reagent, cell samples were plated out at $2x10^5$ cells/ml, 1 ml/well on 24 well culture plates and maintained in complete DMEM with serum for 48 hours prior to washing and extraction using tri-reagent.

PCR was carried out on the cDNA, as in section 2.5.4 after optimizing for $MgCl_2$ concentration and temperature. These conditions are shown in *Table 3.1*. The sequences and product size of the primers used are shown in *Table 3.2*.

Primers	Annealing temperature	$MgCl_2$ concentration
EP2	57 °C	1.1 mM
EP4	57 °C	1.1 mM
FP	63 °C	1.6 mM

Table 3.1: Optimum annealing temperature and $MgCl_2$ concentration for EP2, EP4 and FP receptor primers.

Product	Primer	Sequence	Product size
EP2	forward	GCAGTACGTCCAGTACTGCC	473 bp
	reverse	TCCGACAACAGAGGACTGAACG	
EP4	forward	CCTTCTACACGCTGGTATGTGG	451 bp
	reverse	ATGAACTGGCGGTGCATGCG	
FP	forward	CACAACCTGCCAGACGGA	490 bp
	reverse	CGACGCCTGAATTTTATA	

Table 3.2: Sequence and product size for prostaglandin receptor primers.

The programme was run for 35 cycles. Products were electrophoresed on a 1.5 % agarose gel at 100 V for 60 min and bands were visualized under UV light and photographed. Plasmid pcDNAI/Amp, containing human EP2, EP4 or FP receptor cDNA (generously donated by Mark Abramovitz, Merck Frosst, Canada Ltd, Kirkland, Quebec, Canada) was used as a positive control.

3.2.2 Localisation of PG Receptors and PGDH in the Human Fetal Membranes, Decidua, Placenta and Myometrium at Parturition

The receptors were localised by immunohistochemistry using specific polyclonal antibodies for the receptor of interest. Tissue was collected and fixed as in section 2.1.2, and cells as in section 2.4.1. The tissue groups used were term active labour and elective

caesarean section for fetal membranes, decidua, myometrium and placenta. Preterm tissue was also used, which was a kind gift from Dr. Inass Osman, Glasgow Royal Infirmary. This tissue consisted of fetal membranes and myometrium in non-labouring and labouring groups. n=5 for every group investigated. The tissue was immunostained using the antibodies as shown in *Table 3.3*:

Primary Antibody	Type	Secondary Antibody
EP2	rabbit polyclonal	donkey anti-rabbit IgG
EP4	rabbit polyclonal	donkey anti-rabbit IgG
FP	rabbit polyclonal	donkey anti-rabbit IgG
PGDH	rabbit polyclonal	donkey anti-rabbit IgG

Table 3.3: Antibodies used for immunlocalisation of prostaglandin receptors and PGDH.

3.3 Results

3.3.1 PG Receptor mRNA

Messenger RNA for the EP2 receptor was detected in none of the amnion, chorio-decidua, placenta or JEG3 cell samples. EP4 mRNA was found in chorio-decidua and placenta samples, with a trace in amnion, and FP mRNA in chorio-decidua and placenta samples (*Figure 3.1*).

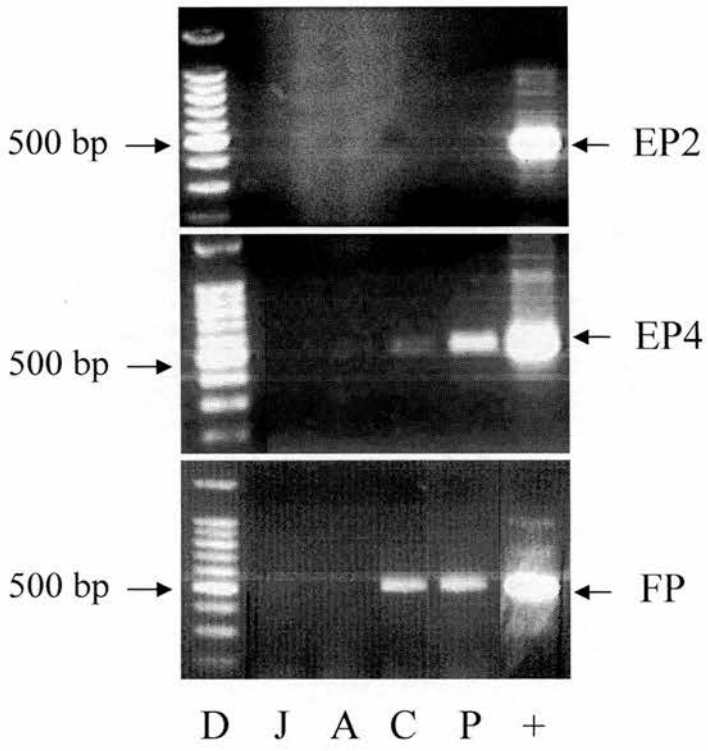


Figure 3.1: RT-PCR gels photographed under UV light showing the presence of prostaglandin receptors EP2, EP4 and FP mRNA in: JEG3 cells (J); amnion (A); chorio-decidua (C); placenta (P). D; DNA ladder, +; positive control.

3.3.2 Localisation of PG Receptors and PGDH in the Human Uterus at Parturition

This immunohistochemistry detected the presence of EP2, EP4, FP and PGDH in the fetal membranes, decidua, placenta, myometrium and JEG3 cells (see *Figures 3.1-3.7* for representative photomicrographs). The immunolocalisation of all three receptors and PGDH was highly consistent throughout all of the samples. There appeared to be no obvious differences in localisation of the receptors between labour and non-labouring samples, and between term and preterm samples. The predominant staining of the receptors was found on the plasma membrane in most cell types. Some nuclear

localisation of the receptors was also obvious from the experiments. There also appeared to be cytoplasmic staining in some cells.

Fetal membranes: The three prostaglandin receptors and PGDH were all localised to the amnion epithelium, with some staining also in cells of the amnion fibroblast layer. In the chorion, they were predominantly found in the trophoblast layer. Staining here of the receptors was clearly mainly in the plasma membrane with some also in the cytoplasm. The receptors and PGDH were also found in cells of fibroblast like morphology in the reticular layer. The same pattern of distribution and localisation was found in term (*Figure 3.2*) and preterm (*Figure 3.3*) fetal membrane tissue sections, except that in the amnion epithelium, FP receptor was not present in preterm samples (labouring or non-labouring), and the EP4 receptor was not present in labouring preterm samples.

Decidua: EP2, EP4, FP and PGDH were found in the glandular epithelial cells of the decidua and also decidual stromal cells and vascular endothelial cells (*Figure 3.4*). There is no evidence of any staining in leukocytes seen in the vessels.

Placenta: The three receptors and PGDH were predominantly visualised in the syncytiotrophoblasts of the placenta (*Figure 3.5*). Cytotrophoblasts appear negative for the receptors and PGDH, as do the endothelial and fibroblast cells of the villous core tissue, with the exception of some PGDH positive staining of stromal core tissue. Extravillous trophoblast cells also appear negative. Fetal and maternal leukocytes appeared negative.

Myometrium: Receptors and PGDH were located in the smooth muscle cells of the myometrium in term (*Figure 3.6*) and preterm (*Figure 3.7*) samples. The staining for EP2, EP4 and FP in these cells appears to be principally localised to the nuclear membrane, except for FP in preterm samples.

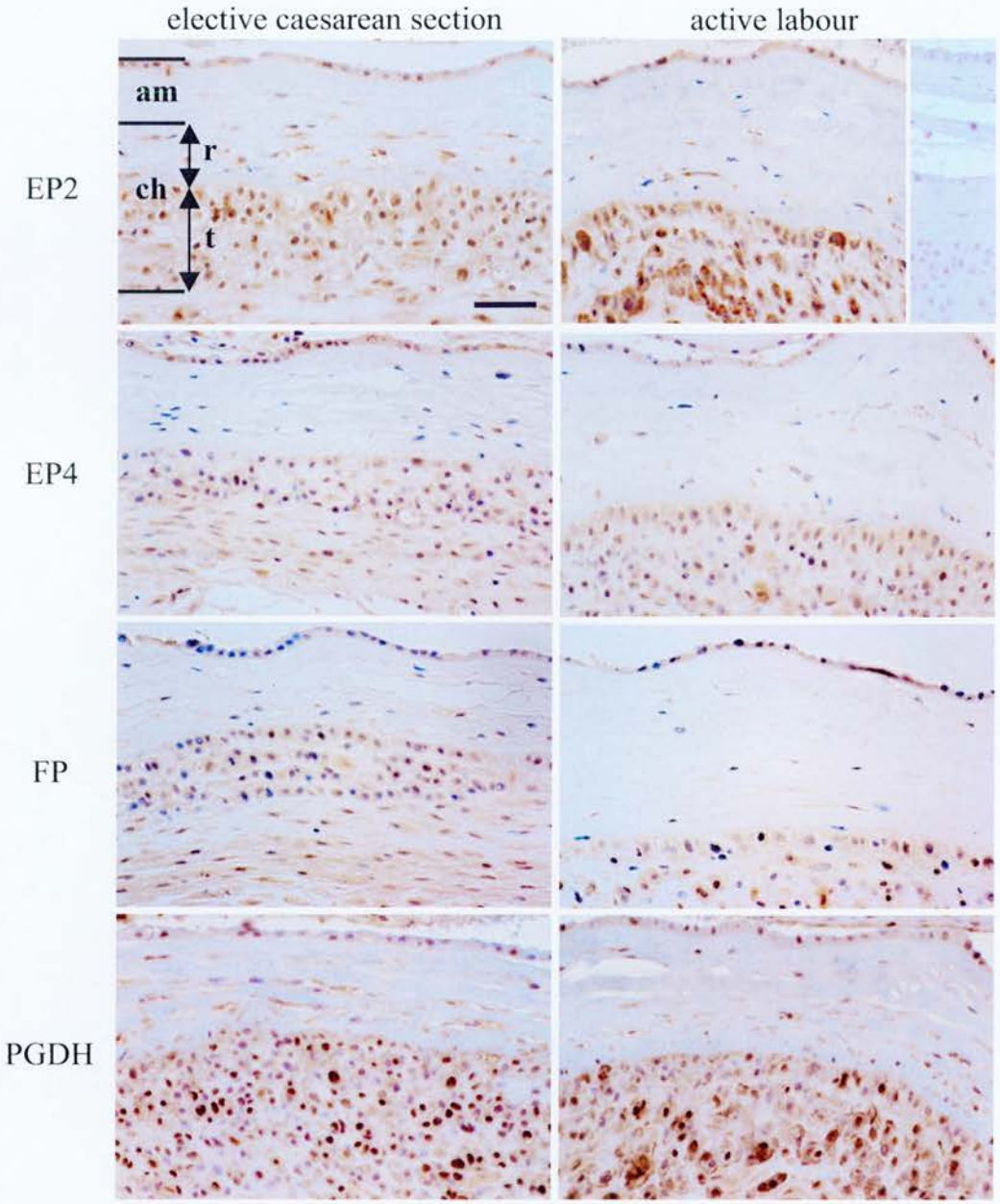


Figure 3.2: Immunolocalisation by DAB detection of EP2, EP4 and FP in sections of fetal membranes with adherent decidua, collected from term active labour births and elective caesarean sections. am, amnion; ch, chorion; dec, decidua; r, reticular layer of chorion; t, trophoblast layer of chorion. All photomicrographs are taken at the same magnification. The scale bar represents 50 μm. Top right shows representative negative control staining of fetal membrane, showing no non-specific staining.

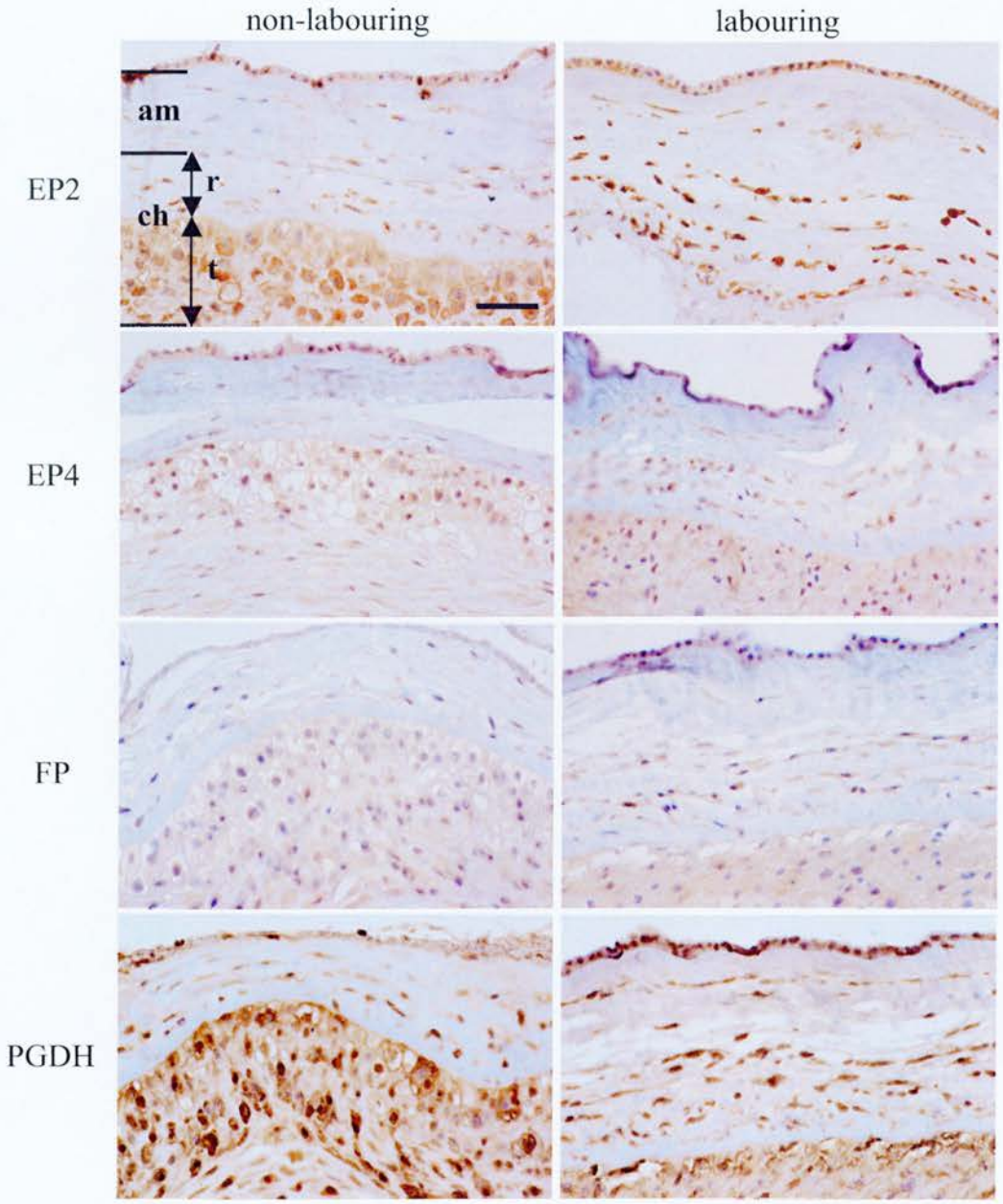


Figure 3.3: Immunolocalisation by DAB detection of EP2, EP4 and FP receptors in sections of fetal membranes collected from preterm labouring and non-labouring births. All photomicrographs are taken at the same magnification. The scale bar represents 50 μm.

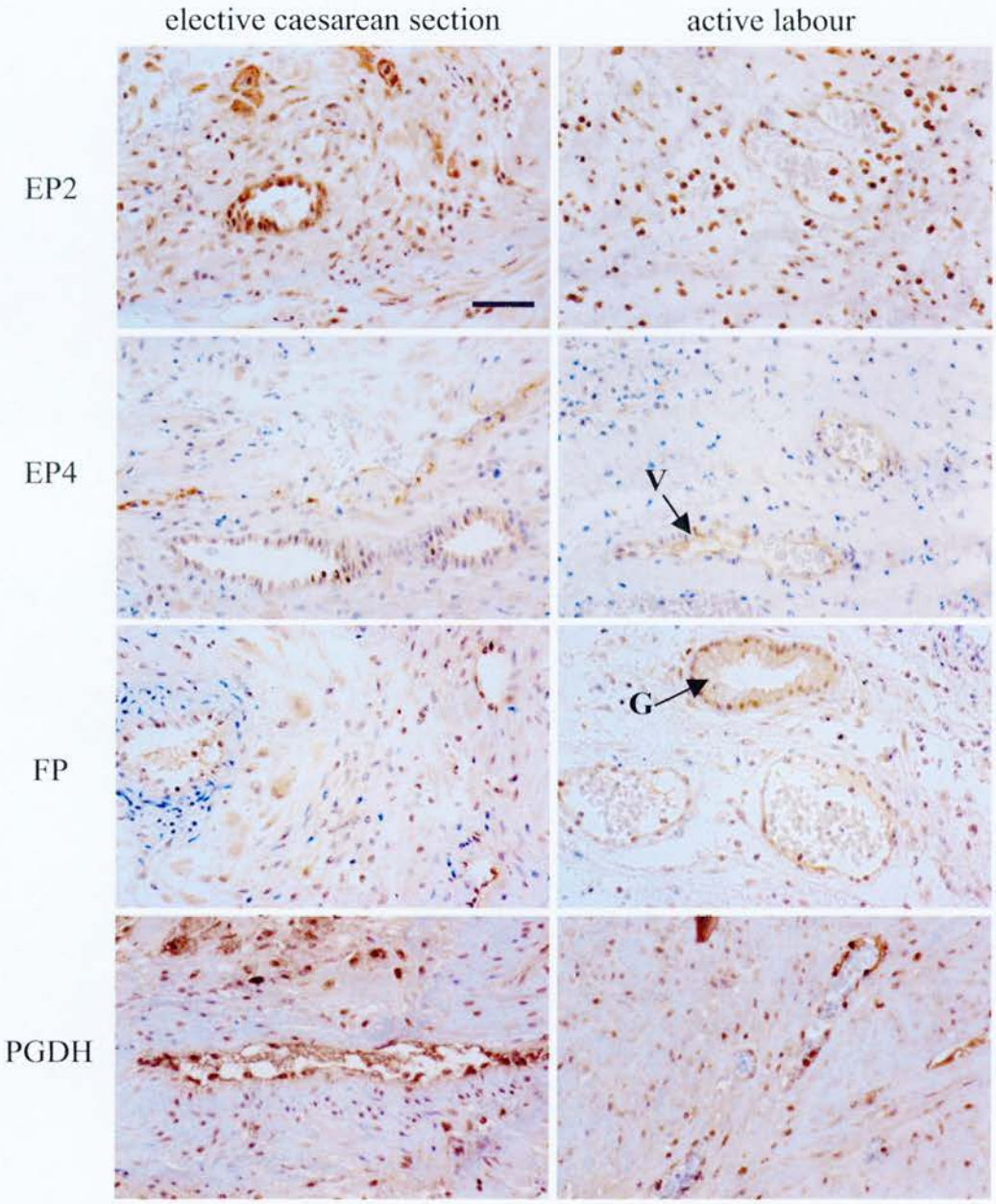


Figure 3.4: Immunolocalisation by DAB detection of EP2, EP4 and FP in sections of decidua collected from term active labour births and elective caesarean sections. All photomicrographs are taken at the same magnification. The scale bar represents 50 μm.

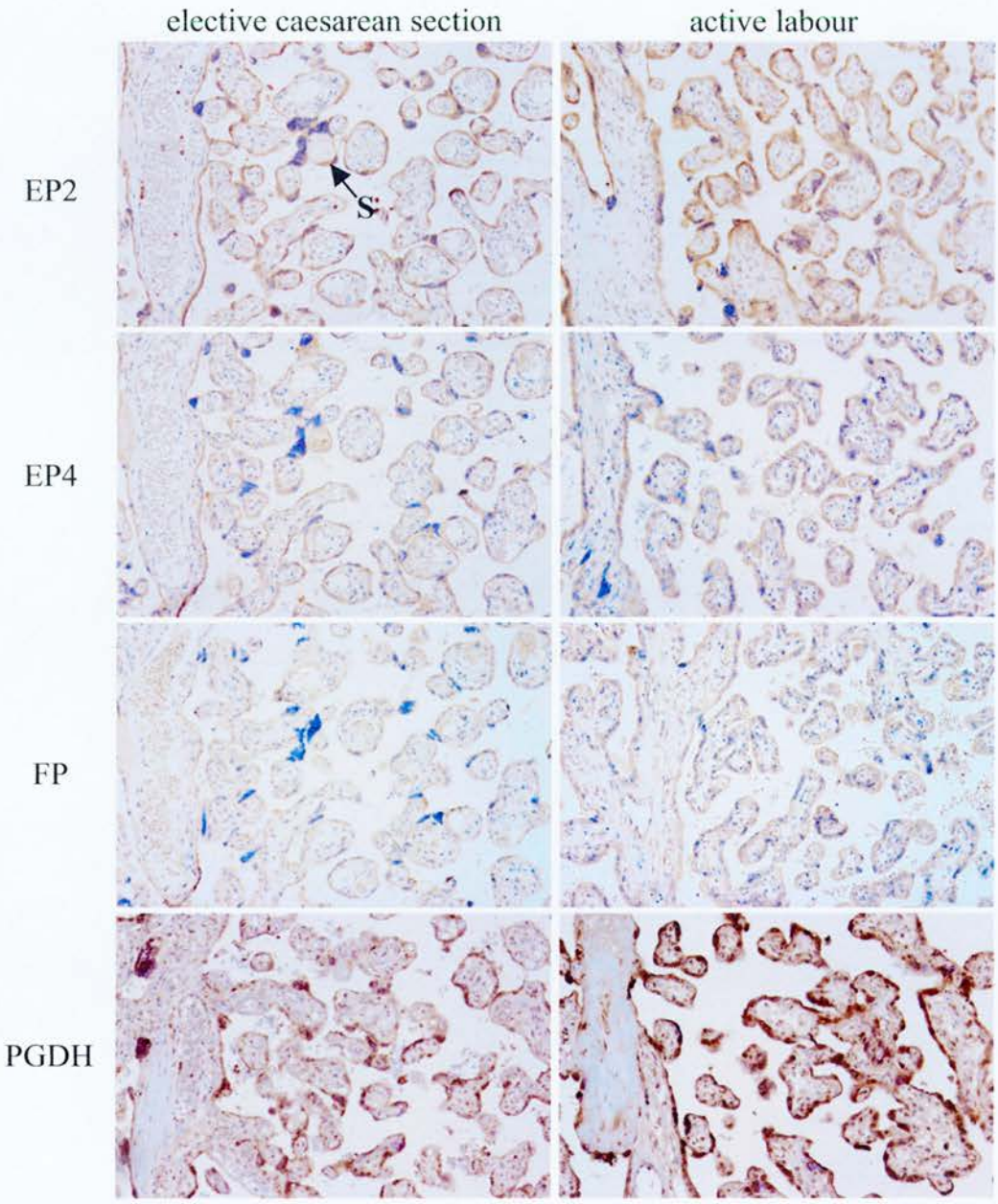


Figure 3.5: Immunolocalisation by DAB detection of EP2, EP4 and FP in sections of placenta collected from term active labour births and elective caesarean sections. s = syncitiotrophoblast All photomicrographs are taken at the same magnification. The scale bar represents 100 μm.

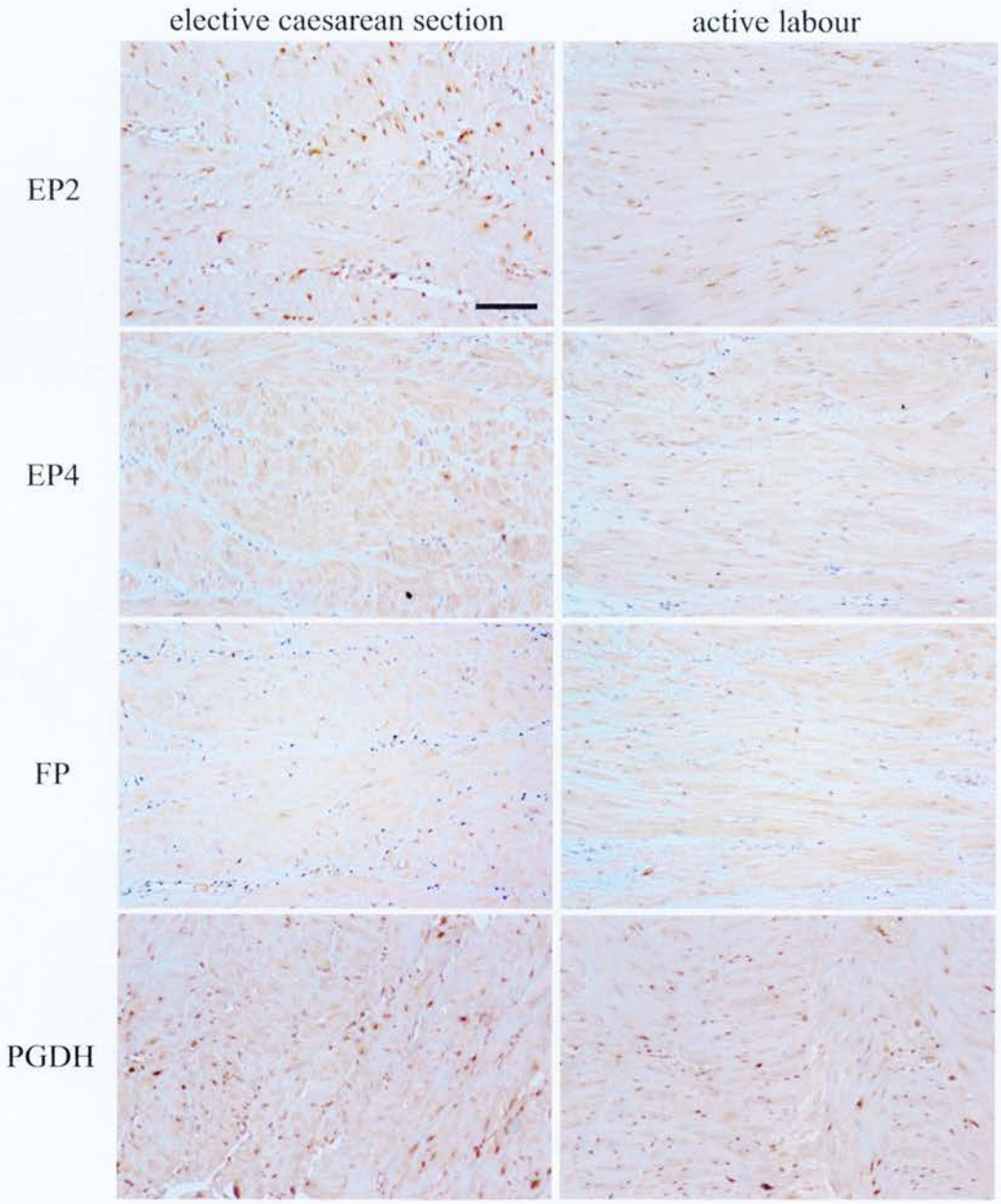


Figure 3.6: Immunolocalisation by DAB detection of EP2, EP4 and FP in section of myometrium collected from term active labour births and elective caesarean sections. All photomicrographs are taken at the same magnification. The scale bar represents 100 μm.

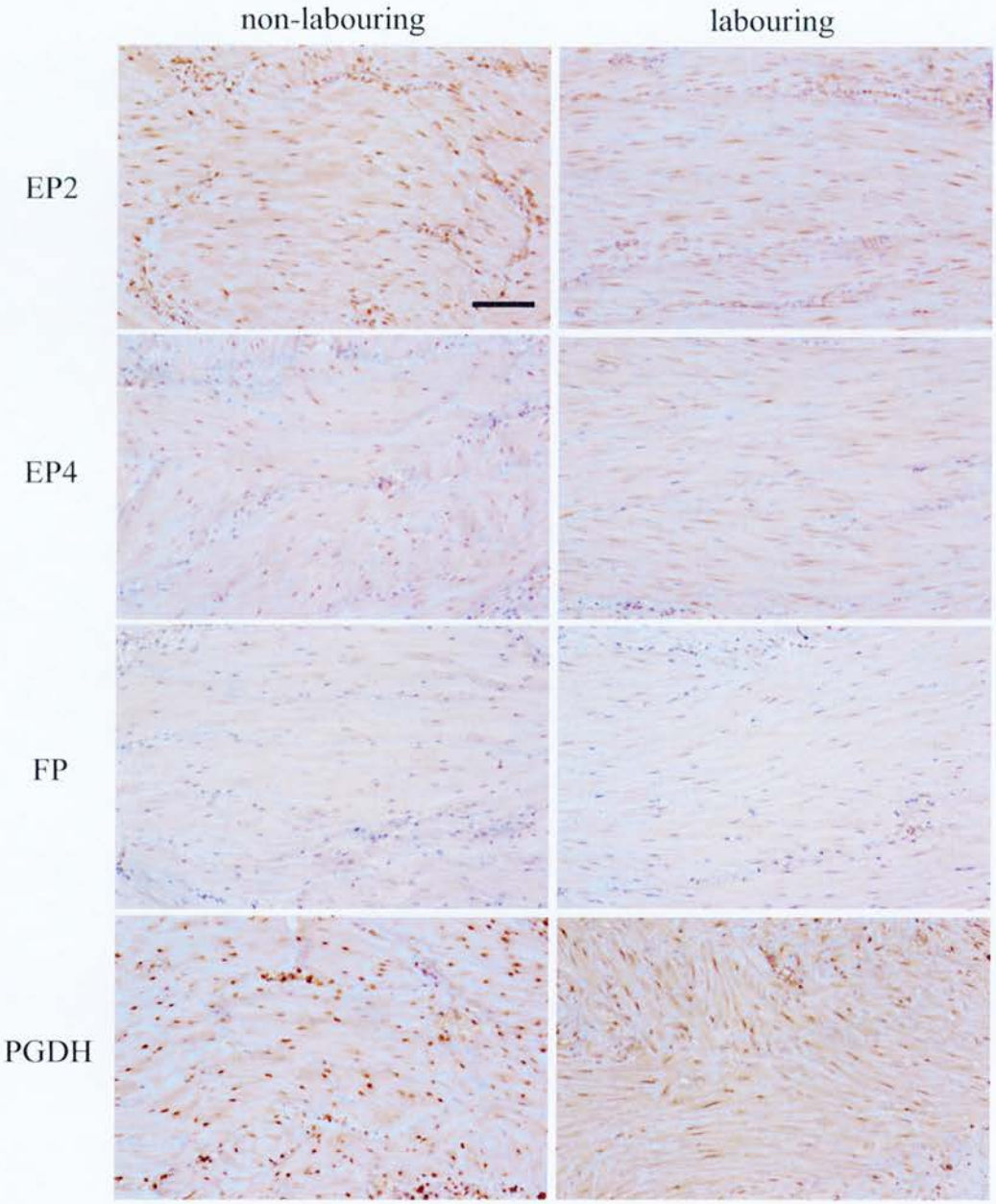


Figure 3.7: Immunolocalisation by DAB detection of EP2, EP4 and FP receptors in sections of myometrium collected from preterm labouring and non-labouring births. All photomicrographs are taken at the same magnification. The scale bar represents 100 μm.

JEG3 choriocarcinoma cells: EP2, EP4, FP and PGDH were all detected in these cells (*Figure 3.8*). All receptors were chiefly located in the plasma membrane, with some partial cytoplasmic staining throughout. EP2 was also localised to the nuclear membrane.

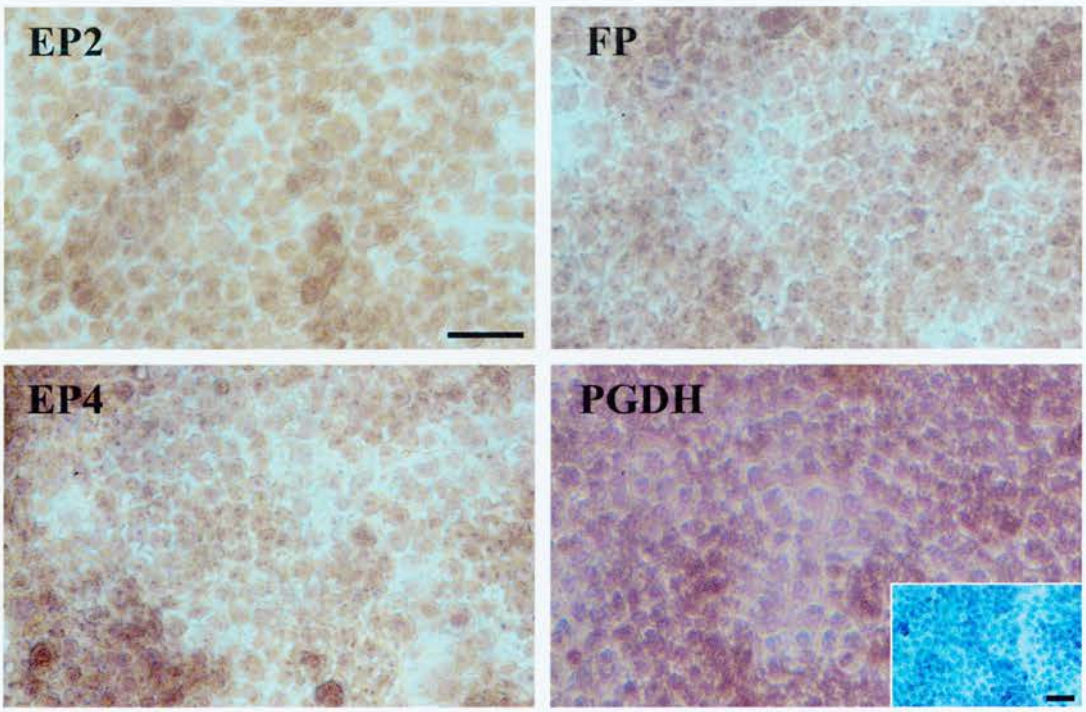


Figure 3.8: Immunolocalisation by DAB detection of EP2, EP4, FP and PGDH in JEG3 cells. Inset, -ve control staining. All photomicrographs are taken at the same magnification. The scale bar represents 50 μm.

3.4 Conclusions

In summary, the EP2, EP4, FP receptors and PGDH were localised to the amnion epithelium and fibroblast layer, the chorion reticular and trophoblast layers, glandular epithelial, vascular endothelial, and stromal cells of the decidua, syncitiotrophoblasts of the placenta, and smooth muscle cells of the myometrium. From this, it can be understood that these are the sites of prostaglandin action and their direct effect within these tissues.

It is unclear why the PCR results and the immunohistochemistry results do not reflect each other. It is possible that the mRNA levels of some of the receptors were too low for detection by standard PCR in the samples in which they were not detected. Other experiments in the laboratory involving Taqman quantitative RT-PCR did detect the presence of EP2 in JEG3 cells. As shown in the next chapter, these cells elicit responses through the EP4 and FP receptors implying that they are present, and the amnion shows a response through the EP2 receptor, which has not been detected by PCR here either. Western blotting was also carried out to try and clarify the presence of the receptor proteins in tissue and cell samples. However, the antibody used proved unspecific, and was therefore deemed unsuitable. That the receptors have all been detected in the tissue and cell-line using immunohistochemistry does indicate their presence in these samples.

Although the receptors are transmembrane, it is possible that they are being detected in the cytoplasm at their site of synthesis, in endoplasmic reticulum, golgi apparatus, or in a vesicle during their trafficking to the cell membranes. The staining viewed in the cytoplasm could also possibly be non-specific. To further clarify the localisation of the prostaglandin receptors, in-situ hybridisation studies to examine receptor mRNA with the tissue sections used, would have been useful. Also, although no detectable changes in levels of prostaglandin receptors was observed between the different groups in the immunohistochemistry studies, to be sure of this the receptor levels would actually have to be quantified. The relative receptor mRNA levels could have been analysed using

Taqman quantitative PCR, had preterm tissue, and tissue from term spontaneous labour been available at the time to extract RNA and create a comparative study. If the Western blotting had proved successful, this could have also been used to quantify receptor protein extracted from tissue in the various groups.

Inter-species differences and limitations in the suitability of animal models restrict research into this area, though there have been results published with regard to prostaglandin receptors in species other than the human. In the baboon, EP2 receptor has been detected in the myometrium, decidua and cervix (Smith et al 1998, Smith et al 2001a, Smith et al 2001b). EP2 and EP4 have also been detected in the mouse uterus (Katsuyama et al 1997). Recently, it was shown that EP2 and FP, but not EP4 were present in bovine fetal membranes (Arosh et al 2004).

The studies in this chapter showed few gestational or labour induced changes in prostaglandin receptor distribution. However, it was seen that the FP receptor was not detected in the amnion epithelium or myometrial nuclear membrane in preterm tissue, whereas it was in term samples. It is possible that the FP receptor is up-regulated with gestational age in these cells. The EP4 receptor was not seen in the amnion epithelium of preterm labouring samples, while it was observed in preterm non-labouring tissue and term samples. A reason for this is not clear. Other literature indicates gestation and labour associated changes in prostaglandin receptor expression occur. It was found that EP3 and FP mRNA levels in the human myometrium were down-regulated during pregnancy, possibly playing a role in the relaxation of the myometrium, thus aiding the maintenance of pregnancy (Matsumoto et al 1997b). EP2 levels can also be seen to decrease with advancing gestational age, implying that these too are involved in uterine quiescence (Leonhardt et al 2003). In the baboon decidua, EP2 expression decreased with labour, which suggests an adenylate-cyclase-mediated effect may inhibit parturition-related processes in decidua (Smith et al 2001b). This parallels decreased EP2 receptor expression in myometrium and cervix (Smith et al 1998).

There was positive nuclear staining of prostaglandin receptors observed from the immunolocalisation studies. This suggests a role for intracrine prostaglandin signalling mechanisms in the nucleus. As well as GPCRs, PPARs are also presumed to be targets of prostaglandins and other eicosanoid hormones. PPARs are members of the nuclear receptor superfamily of transcription factors that include steroid receptors. Three subtypes have been identified - α, δ, and γ. PGE_2 and $PGF_{2\alpha}$ are both naturally occurring ligands for PPARα (Forman et al 1997), and PGE_2 also binds to PPARγ (Ferry et al 2001). There is little information to date on the role of PPARα in pregnancy and parturition. PPARα deficient mice have normal fertility (Lee et al 1995) so this receptor apparently does not have a major role in normal reproductive function. The finding of PPARα mRNA in term amnion, chorio-decidua and placenta (Eykholt et al 2004, Helliwell et al 2004) (Wang et al 2002) suggests there is a role for this receptor in gestational tissue, however what this is remains unclear. PPARγ has been found to be important in placental development (Barak et al 1999). It is expressed in human placental tissues at various stages of gestation (Helliwell et al 2004) and has been immunolocalised to the nuclei of the syncytiotrophoblast, cytotrophoblast and endothelial cells of the term placenta (Schaiff et al 2000). It has also been identified in human chorio-decidua and JEG3 cells (Keelan et al 1999b, Marvin et al 2000). There were also small amounts of PPARγ in a few amnion samples (Marvin et al 2000). Recent data has detected PPARγ in term labouring and non-labouring amnion, chorio-decidua and placenta, and shown that PPARγ levels with the onset of labour, as COX-2 levels increase, and a positive feedback mechanism of prostaglandin action during parturition through this receptor has been suggested (Dunn-Albanese et al 2004).

The localisation of the prostaglandin receptors correlates well with the sites of prostaglandin metabolism. Immunolocalisation of PGDH, has shown that the tissue distribution of this enzyme is the same as these receptors, with a prominent location in the chorion trophoblast cells. This corresponds to previous reports on the distribution of PGDH which shows the enzyme in amnion, chorion and decidua with the order of

activity as follows: chorion > decidua > amnion (Okazaki et al 1981). Other groups have also shown high chorion PGDH levels, with localisation to the trophoblast layer (Cheung et al 1990, van Meir et al 1997). COX enzymes are expressed in the amnion, chorion and decidua, with highest levels being in the amnion (Cheung et al 1990, Gibb & Sun 1996, Okazaki et al 1981, Rose et al 1990, Teixeira et al 1994). The amnion is thus the primary site of PG synthesis, synthesizing principally PGE_2. So, overall, PGE_2 and $PGF_{2\alpha}$ output is greater in the amnion, than in the chorion and decidua (Cheung et al 1990). Only very small amounts of these prostaglandins will cross the fetal membranes without being metabolized (Roseblade et al 1990). COX enzymes and PGDH were also found in the placenta (Cheung et al 1990, Greystoke et al 2000, Jarabak 1972, Macchia et al 1997, Schoof et al 2001) and myometrium (Giannoulias et al 2002, Moonen et al 1984).

Changes in expression of the enzymes involved in prostaglandin biosynthesis occur with advancing gestational age and labour. Two isoforms of the COX enzyme – COX-1 and COX-2 are known to be involved in catalyzing the production of prostaglandins. COX-1 is constitutively expressed, and COX-2 is known to be inducible (Morita 2002). COX-2 mRNA increases at the onset of labour in the amnion and chorion in coordination with one another implying a concerted mechanism of regulation in the fetal membranes at both term and preterm (Mijovic et al 1999, Mijovic et al 1997). There are no reported COX changes in the decidua with labour (Hirst et al 1998, Mijovic et al 1999), however COX-2 mRNA has been shown to be up-regulated in chorio-decidual tissue with labour (Slater et al 1998). Evidence suggests that although PGDH levels do not change in the amnion and decidua (Skinner & Challis 1985), in the chorion, mRNA expression and activity of PGDH decrease in human labour, at term and preterm (Patel & Challis 2002, Sangha et al 1994). The combination of increase in synthesis and decrease in degradation of prostaglandins during labour gives rise to overall higher intrauterine prostaglandin levels at term and preterm.

In the placenta, reports of COX-2 expression increasing with gestation have been published, with no changes in COX-1 levels, and no labour induced changes in either COX isoform in sheep (Gibb et al 1996, Rice et al 1995). In the human placenta, PGES activity decreases with the onset of labour – suggesting that PGE_2 has been maintaining uterine quiescence and its degradation is associated with initiating labour (Alfaidy et al 2003). Placental PGDH mRNA levels increase during pregnancy, implying a role in delaying prostaglandin induced uterine contractions presumably by degrading $PGF_{2\alpha}$ (Schoof et al 2001).

In the human myometrium, COX-1 and COX-2 levels are greater in the pregnant compared to the non-pregnant state (Moonen et al 1984), although labour associated changes are not clear. They have been reported to increase (Erkinheimo et al 2000), decrease (Zuo et al 1994) and stay the same (Giannoulias et al 2002, Moore et al 1999). A significant decrease in PGDH protein levels and activity is found in the myometrium with labour at term and preterm (Giannoulias et al 2002). Also, in the cervix, PGDH expression was found to be higher in the unripe than ripe condition, irrespective of gestation time. COX-1 and COX-2 expression did not differ. This suggests that increased levels of prostaglandin output are associated with cervical ripening at term and preterm (Tornblom et al 2004).

Putting together the observed changes in prostaglandin biosynthesis and catabolism, and receptor expression in uterine and gestational tissue during pregnancy and labour can give an indication of proposed prostaglandin target sites at these times. The amnion produces large quantities of prostaglandins, particularly PGE_2, with production increasing during labour. The EP2, EP4 and FP receptors are present at term and preterm labour in labouring and non-labouring amnion. Thus, the amnion is a likely target site for prostaglandin action. The chorion trophoblast represents a target site also, as the receptors are expressed here, and the chorion also is a place of prostaglandin degradation. This paracrine and autocrine action of prostaglandins on the fetal

membranes may lead to their rupture at term or preterm by stimulating production and activation of MMP-2 and MMP-9 (McLaren et al 2000b, Ulug et al 2001).

Chapter 4: Signalling Pathways via Prostaglandin Receptors in Term Human Fetal Membranes

4.1 Introduction and Aims

Prostaglandin ligand-receptor interactions play a key role in many reproductive functions, including parturition. The intracellular pathways which these receptors trigger when activated have been investigated (Ashby 1998) as discussed in section 1.4. Once activated, these intracellular pathways can activate transcription factors and control gene expression, and they can also activate various proteins via mechanisms such as phosphorylation. The prostaglandins have effects on various factors during pregnancy and parturition, for example cytokines and MMPs. These effects are mediated through their receptors by activating intracellular pathways.

There is little published data on the involvement of PG stimulated pathways in the regulation of cytokines or MMPs in human uterine and gestational tissue. More roles of intracellular mediators have been reported in animal models however. Levels of MMP-2 and MMP-9 have been found to be regulated in part by a PGE_2-cAMP dependent mechanism in the rat uterus and cervix (Lyons et al 2002), with prostaglandins stimulating MMP-2 and MMP-9 in labouring tissues. cAMP has also been shown to play a role in uterine quiescence (Price & Bernal 2001). Further to this, it has been found that $G_{\alpha s}$ subunit expression in the human myometrium is greater in the pregnant compared to non-pregnant state and so is its associated increase in adenyl cyclase activity. However, upon spontaneous labour, levels fall back to the non pregnant state. This suggests that cAMP contributes to prolonged relaxation of the uterus during gestation and down-regulation of $G_{\alpha s}$ would decrease the relaxing effect exerted by cAMP and may be a triggering mechanism for the initiation of labour (Europe-Finner et al 1994).

There is a lot of evidence for a role of inositol phosphates in myometrial contractions. Phosphoinositide breakdown is thought to be important in regulating a variety of transmembrane signal transduction events in the action of oxytocin during smooth muscle contraction. Oxytocin, PGE_2 (Okawa et al 1993) and $PGF_{2\alpha}$ (Maka et al 1993, Okawa et al 1993) have all been found to stimulate IP production in pregnant and non-

pregnant rabbit myometrium. Contradictory to this, PGE_2 and $PGF_{2\alpha}$ have both been found to have no effect on phosphoinositide hydrolysis in human smooth muscle, though they were shown to stimulate arachidonic acid release contributing to prostaglandin production, and Ca^{2+} which is involved in contraction of the myometrium (Schrey et al 1988). Another study showed PGE_2 and $PGF_{2\alpha}$ stimulated cAMP generation and inositol phosphate release in rabbit myometrium and that PLC activation was coupled not only to $PGF_{2\alpha}$ but also to PGE_2 receptors and could be correlated with contractions induced by $PGF_{2\alpha}$ and PGE_2 (Goureau et al 1992).

MAPKs have been shown to mediate MMP-9 expression in rat arterial smooth muscle cells (Cho et al 2000). It is not known whether a similar control is exerted in myometrial smooth muscle and if this can be activated by prostaglandins. It is evident that MAPK plays a role in uterine quiescence. In the rat myometrium, $PGF_{2\alpha}$ can activate the MAPK cascade through the $\beta\gamma$ subunit of a G-protein which possibly involves a receptor tyrosine kinase, and ultimately leads to cell contraction (Ohmichi et al 1997).

Having now localised the prostaglandin receptors, EP2, EP4 and FP within human fetal membranes, decidua, placenta, myometrial tissue and JEG3 cells, the aims of this chapter are to investigate the signalling pathways which are activated upon prostaglandin binding to these receptors. As EP2 and EP4 are linked to $G_{\alpha s}$, changes in cAMP levels will be examined on treatment with PGE_2. The FP receptor is $G_{\alpha q}$ linked, so total inositol phosphate levels will be monitored upon addition of $PGF_{2\alpha}$. Both receptors have been shown to activate the MAPK pathway so phosphorylation of MAPK will be determined following treatment with PGE_2 and $PGF_{2\alpha}$. Selective inhibitors of MEK, PLC and EGFR kinase will also be added to further elucidate the pathways involved in prostaglandin synthesis. Specific antagonists of the EP2, EP4 and FP receptors will also be utilised to clarify the receptors involved in these signalling pathways. The proposed pathways to be activated are shown in *Figure 4.1*.

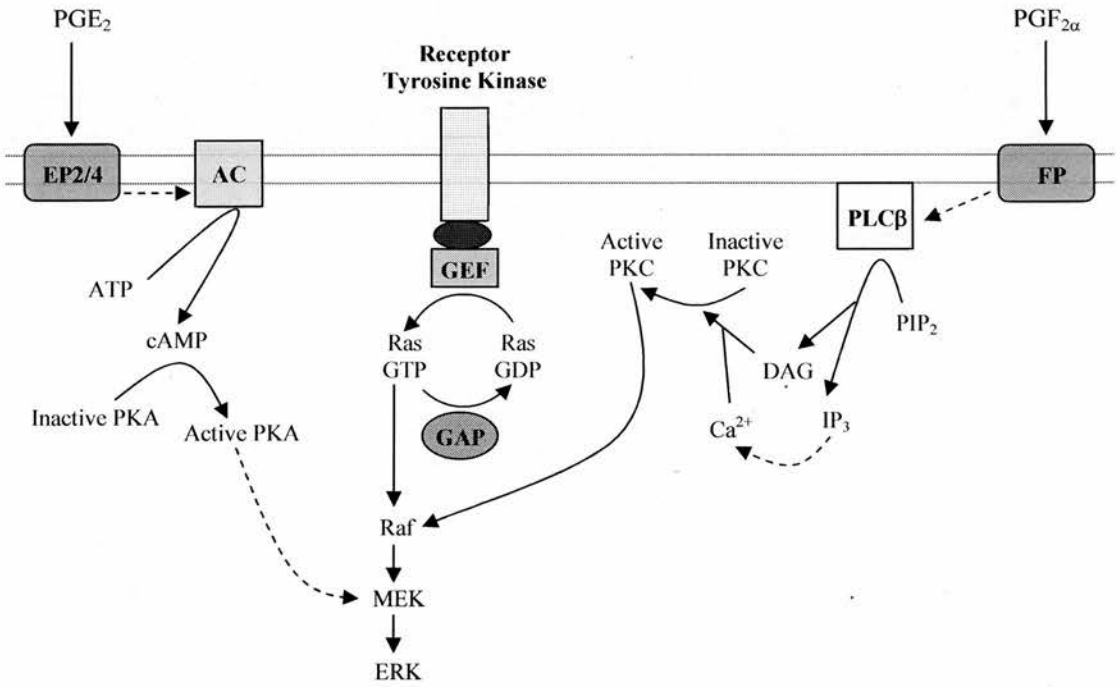


Figure 4.1: Proposed mechanisms of prostaglandin signalling via EP2, EP4 and FP receptors. AC; adenyl cyclase, PKA; protein kinase A, PKC; protein kinase C, PLCβ; phospholipase Cβ, DAG; diacylglycerol, PIP2; phosphatidylinositol 4,5-biphosphate, IP3; inositol 1,4,5-triphosphate, ERK; extracellular signal regulated kinase, MEK; ERK kinase, GTP; guanosine triphosphate, GDP, guanosine diphosphate, GEF; guanine exchange factor GAP; GTPase activating protein.

4.2 Methods

4.2.1 PGE_2 Effect on cAMP Production

4.2.1.1 Fetal Membrane Time Course Study

Fetal membranes were collected as in section 2.1.1 and cultured as in 2.1.2. Prior to cutting, tissue was steeped in steeping buffer (appendix 1.1.2) containing 10 μM indomethacin for 2 hours. After cutting, explants of amnion and chorio-decidua were mounted and incubated in 24 well plates in complete RPMI (appendix 1.1.3) containing 10 μM indomethacin (a COX inhibitor) and 1 mM 3-isobutyl-1-methylxanthine (IBMX) (a phosphodiesterase inhibitor) for 1 hour. Following this, PGE_2 was added into the wells to a final concentration of 100 nM for 0, 5, 10 or 15 minutes. The PGE_2 was mixed in by swirling. After the treatment time, the tissue was removed using forceps and dipped briefly into DPBS twice to wash. Then it was put into 0.5 ml ice cold lysis buffer 1B (cAMP assay kit) in an eppendorf tube and kept on ice until being homogenised (section 2.7.1.4) and frozen at -70 °C until assaying. For this experiment, n=4 patients, and each treatment group was replicated 3 times for each patient.

4.2.1.2 JEG3 Cell Time Course Study

JEG3 cells were diluted to a concentration of 2 x 10^5 cells/ml and plated out into 6 well plates with 3 ml/well. They were left to adhere for 24 hours, and then the medium was aspirated and replaced with serum free DMEM containing 10 μM indomethacin for a following 24 hours. The medium was then aspirated and replaced with serum free DMEM containing 10 μM indomethacin and 1 mM IBMX for 1 hour. After this, PGE_2 was added and mixed into the wells to a final concentration of 100 nM over a time course of 0 to 35 min. The medium was then aspirated and the cells washed twice with DPBS before adding 300 μl lysis reagent 1B (cAMP assay kit) to each well. The plates were then rocked on ice for 10 min, then the cells scraped and the contents of each well pipetted into an eppendorf tube. This was centrifuged at 10, 000 g for 20 min at 4 °C, the

supernatant removed and kept frozen at -70 °C until a cAMP assay was carried out. For this experiment, n=2 and within each individual experiment, 3 replicates were used of each treatment.

4.2.1.3 Amnion Antagonist Study

Amnion was collected and treated as in section 4.2.1.1 until the IBMX incubation stage. Here, at this step, in addition to IBMX, the tissue was incubated with or without selective receptor antagonists. Antagonists were: 10 μM AH6809 (for EP2); and 300 nM ONO-AE2-227 (for EP4) added. Following this, PGE_2 was added into the wells to a final concentration of 100 nM for 10 min. The treatment groups were: control (vehicle (DMSO) + no PGE_2), vehicle + PGE_2, EP2 antagonist + PGE_2, EP4 antagonist + PGE_2, and EP2 + EP4 antagonists + PGE_2. The tissue was then washed and homogenised as in section 4.2.1.1 and a cAMP assay carried out. For this experiment, n=3 patients, and each treatment group was replicated 3 times for each patient.

4.2.1.4 JEG3 Cell Antagonist Study

The protocol for this experiment was the same as section 4.2.1.2, except selective receptor antagonists were added in addition to IBMX as in section 4.2.1.3. For this experiment, n=2 and within each individual experiment, 3 replicates were used for each treatment.

4.2.1.5 cAMP ELISA

The samples of amnion, chorio-decidua and JEG3 cells prepared as above were assayed for intracellular cAMP content using an enzyme-immunoassay (section 2.6.3). Samples were defrosted to room temperature before use. Amnion samples were used undiluted, chorion samples diluted 1 in 2 and JEG3 cell samples diluted 1 in 10 in lysis buffer 1B to fit into the range of the assay. Each sample was assayed in duplicate and a standard curve was carried out for every 96 well assay plate used.

4.2.1.6 Protein Assay

A protein assay (section 2.6.1) was carried out for every sample which was assayed for cAMP so as to express the cAMP levels in pg/mg of protein. The cAMP levels were then expressed as a percentage of the control (time 0, no PGE_2 treatment). This allowed for variation between patients.

4.2.2 $PGE_2/PGF_{2\alpha}$ Effect on ERK1/2 Phosphorylation

4.2.2.1 Fetal Membrane Time Course Study

Amnion and chorio-decidua were collected as in section 2.1.1 and cultured as 2.1.2. Prior to cutting the explants, tissue was steeped in steeping buffer containing 10 μM indomethacin for 2 hours. After explants were cut and mounted, they were steeped in 1 ml complete RPMI plus either PD98059 to a final concentration of 50 μM, U73122 to a final concentration of 10 μM or the same volume of DMSO as a control, as these inhibitors are diluted in DMSO. After 1 hour incubation, tissue was treated with either 100 nM PGE_2 or 100 nM $PGF_{2\alpha}$ for 5, 10 or 15 min, or left untreated (time 0). After this treatment, the tissue was washed twice in DPBS and then added to 500 μl NP40 lysis buffer (appendix 1.5.1.2). It was then homogenised, centrifuged and sonicated (section 2.7.1.4). The supernatant was kept at -20 °C until protein assay and Western blot analysis. (n=4 different patients, with every treatment replicated 3 times for each patient).

4.2.2.2 JEG3 Cell Time Course Study

Cells were diluted to 2 x 10^5 cells/ml and plated out in 5 ml volumes into Petri dishes. They were left 24 hours, then the medium was replaced with serum free DMEM plus 10 μM indomethacin for a further 24 hours. The medium was aspirated and replaced with serum free DMEM +/- PD98059 to a final concentration of 50 μM, U73122 to a final concentration of 10 μM, or DMSO as a control for 1 hour. Cells were then treated with either PGE_2 or $PGF_{2\alpha}$ to a final concentration of 100 nM for 5, 10 or 15 minutes, or left untreated (time 0). Following this, the medium was aspirated and cells washed twice

with DPBS, before adding 300 μl NP40 lysis buffer. Cells were then scraped and the dish contents collected into a tube and centrifuged and sonicated (section 2.7.1.4) and the supernatant kept at -20 °C until protein assay and Western blot analysis. (n=3 separate and independent experiments).

4.2.2.3 JEG3 Cell Receptor Antagonist Study

The protocol for this study is as section 4.2.2.2, except instead of adding inhibitors for 1 hour prior to PG treatment, receptor antagonists were added. These antagonists were: EP2 (AH6809); EP4 (ONO-AE2-227); and FP (AL8810) added to final concentrations of 10 μM, 300 nM and 100 μM respectively. Samples were then treated with 100 nM PGE_2 or $PGF_{2\alpha}$ for 10 min. Treatment groups were control (vehicle (DMSO) + no PG), vehicle + PGE_2, EP2 antagonist + PGE_2, EP4 antagonist + PGE_2, EP2+EP4 antagonists + PGE_2, vehicle + $PGF_{2\alpha}$ and FP antagonist + $PGF_{2\alpha}$. (n=2 experiments with 2 repeats for each treatment).

4.2.2.4 JEG3 Cell EGFR Kinase Inhibitor Study

The protocol for this study is as section 4.2.2.2, with pre-treatment being with or without 100 nM AG1478. This compound is an inhibitor of EGFR kinase, preventing EGFR from becoming tyrosine phosphorylated, dimerising, and signalling. Treatments were 100 nM PGE_2 or $PGF_{2\alpha}$ for 10 min. (n=2 experiments with 2 repeats for each treatment).

4.2.2.5 Protein Assay

Every sample collected underwent protein assay (section 2.6.1) prior to Western blotting to ensure equal loading of all samples into the gels.

4.2.2.6 Western Blot Analysis for ERK Phosphorylation

Gel electrophoresis was carried out for all samples as described in section 2.7.2.3. 20 μg protein was loaded for each sample. After transfer, and blocking the membranes with BSA, they were incubated in primary antibody (P42/P44 ERK-P) overnight. After

washing, secondary antibody binding and further washing, proteins were detected using ECL (see section 2.7.2.3). The photographic film was exposed to the membrane until sharp, clear bands were observed on developing (approximately 10 min exposure). The bands were then analysed by densitometry using Biorad Quantity One software and levels of phosphorylation expressed relative to control levels (no PG treatment, same inhibitor treatment as sample being compared). Normalising the data like this allowed for any slight background differences between autoradiographs and differences between patients.

4.2.3 IP3 Pathway

JEG3 cells were diluted to 2 x 10^5 cells/ml and plated out into 24 well plates at 1 ml/well. They were left overnight before the medium was aspirated and replaced with DPBS plus tritium (myo-[^{3}H]-inositol at 1 μCi/well). This was left for a further 24 hours, then the medium aspirated and washed once with Buffer A (appendix 1.4.3). Then 500 μl buffer A + 10 mM LiCl was added for 30 min at 37 °C gently shaking. The medium was aspirated and treatment added for 1 hour. Treatments were 10 nM or 100 nM $PGF_{2\alpha}$ diluted in buffer A, with buffer A as a control. This was then aspirated and replaced with 500 μl formic acid on ice and incubated at 4 °C for 30 min. This was then removed from the well and added to a tube and 500 μl Dowex resin added, mixed and allowed to settle. The top layer was aspirated, and the remaining washed with 1 ml water. This was mixed, allowed to settle, the top aspirated and 1 ml 60 mM ammonium formate/5 mM sodium tetraborate added and mixed. After letting this settle and aspirating the top layer, 1 ml of 1 mM ammonium formate/ 0.1 M formic acid was added and mixed and allowed to settle. 0.8 ml of the top layer was transferred to a scintillation tube, scintillation fluid added and the associated radioactivity determined by liquid scintillation counting for 1 min. (n=2 separate and independent experiments, with treatments in triplicate for each).

4.3 Results

4.3.1 cAMP Results

4.3.1.1 PGE_2 increases cAMP production in amnion and not chorio-decidua

Cyclic AMP production in response to PGE_2 treatment in amnion tissue (*Figure 4.2a*) increased significantly after 5, 10 and 15 minutes, with a peak after 10 min. The baseline (control) level of cAMP was 5.23 ± 0.06 pmol/mg protein. The cAMP levels relative to the control were as follows: 5 min = 270 ± 7 %; 10 min = 302 ± 45 %; 15 min – 192 ± 7 %. These results were all significant when compared to time 0. The baseline level of cAMP in the chorio-decidua was 13.93 ± 2.69 pmol/mg protein. cAMP levels also increased in chorio-decidua (*Figure 4.2b*), with changes as follows: 5 min = 140 ± 29 %; 10 min = 154 ± 49 %; 15 min = 136 ± 29%. However, none of these results were found to be significant when compared to the control.

4.3.1.2 PGE_2 increases cAMP production in JEG3 cells

In the JEG3 cell-line, cAMP levels were found to increase on stimulation with PGE_2 treatment (*Figure 4.3*). This increase in cAMP was in a time–dependent manner, with a slight reduction in cAMP levels observed after 15 and 30 min. The baseline cAMP level was 56.23 ± 3.27 pmol/mg protein. The cAMP relative to control levels was as follows: 2 min =145 ± 8 %; 5 min = 150 ± 17 %; 10 min = 179 ± 9 %; 15 min = 134 ± 12 %, 20 min = 243 ± 22 %; 25 min = 293 ± 15 %; 30 min = 260 ± 27 %; 35 min = 302 ± 37 %. These increases were all significant.

4.3.1.3 EP2 antagonist inhibits PGE_2 stimulation of cAMP in amnion

Addition of an EP2 antagonist totally inhibited the cAMP stimulation in response to PGE_2 treatment after 10 min (*Figure 4.4a*) in amnion tissue. An EP4 antagonist had no effect on this response, however a combination of both EP2 and EP4 receptor antagonists totally inhibited the response like EP2 alone. The cAMP levels relative to the control were: no antagonist: 174 ± 28 %; EP2 antagonist = 105 ± 7 %; EP4

antagonist = 179 ± 31 %; EP2 + EP4 antagonist = 106 ± 10 %. The rise of cAMP with PGE_2 and with PGE_2 in combination with the EP4 antagonist were significant when compared with the control, and the EP2, and combined EP2 + EP4 antagonist effects were significant when compared to a 10 min stimulation with PGE_2.

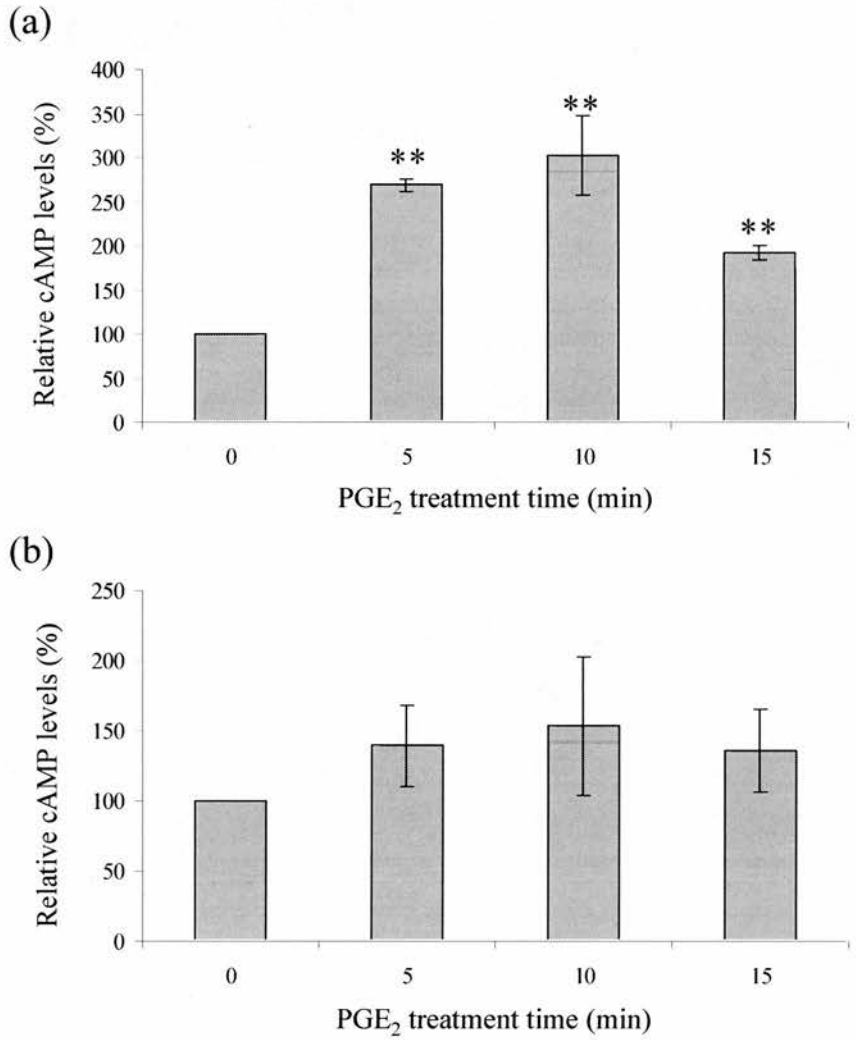


*Figure 4.2: cAMP levels after treatment with 100 nM PGE_2 for 0, 5, 10 and 15 min in (a) amnion and (b) chorio-decidua. * denotes a significance value of $p < 0.05$, ** is $p < 0.01$ compared to the control at time 0. n=4 patients, with each treatment group replicated 3 times.*

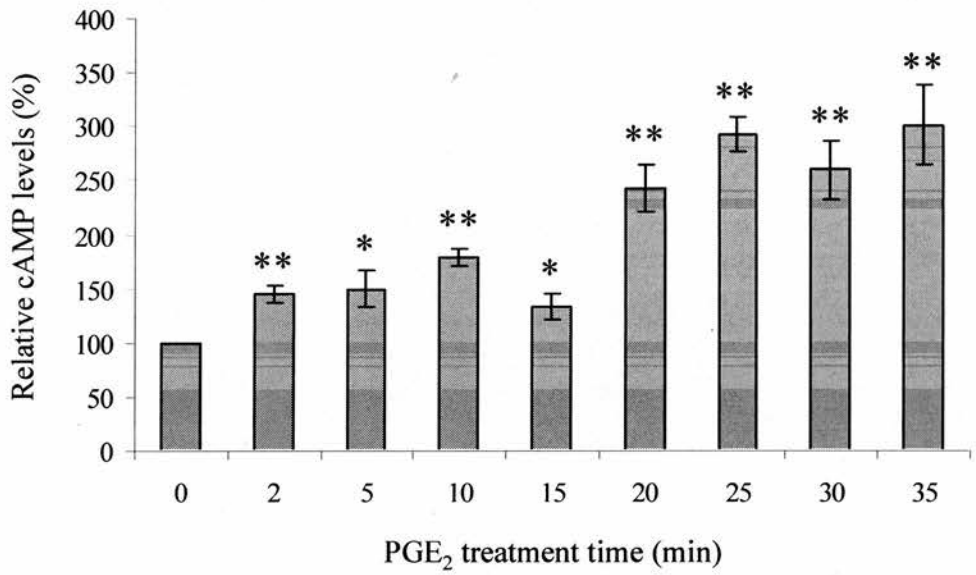


*Figure 4.3: cAMP levels in JEG3 cells after treatment with 100 nM PGE_2 for 0, 2, 5, 10, 15, 20, 25, 30 and 35 min. * denotes a significance value of $p < 0.05$, ** is $p < 0.01$ compared to the control at time 0. n=2, with 3 replicates of each treatment.*

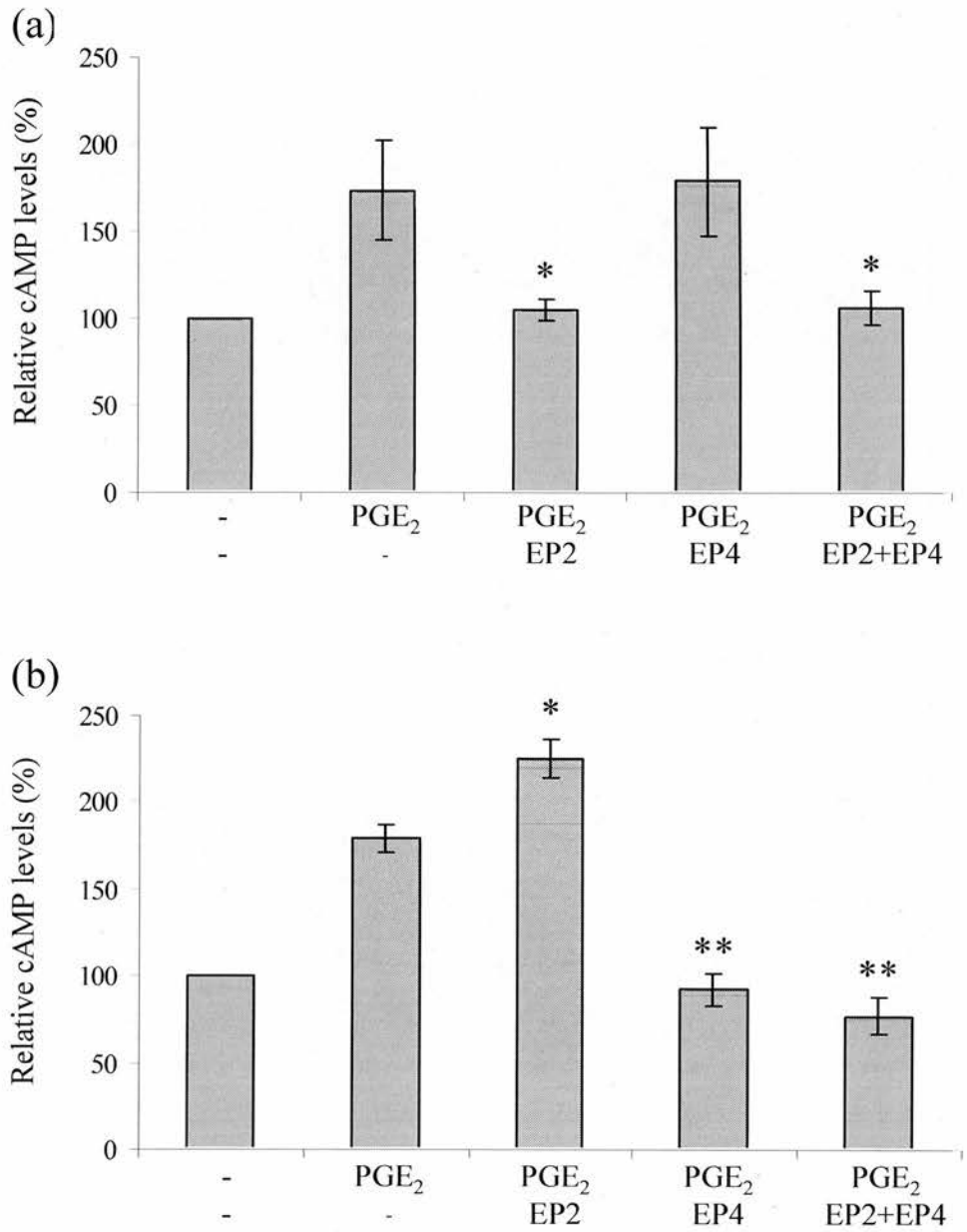


*Figure 4.4: cAMP levels after treatment with 100 nM PGE_2 for 0 and 10 min in the presence or absence of EP2 and EP4 antagonists in (a) amnion and (b) JEG3 cells. * denotes a significance value of $p < 0.05$, ** is $p < 0.01$ compared to the samples with no antagonist.* n=3 patients, with 3 replicates of each treatment.

4.3.1.4 EP4 antagonist inhibits PGE_2 stimulation of cAMP in JEG3 cells

The cAMP levels in response to PGE_2 and EP2 and EP4 antagonists were as follows: PGE_2 alone = 179 ± 9 %; PGE_2 + EP2 antagonist = 226 ± 11 %, PGE_2 + EP4 antagonist = 92 ± 9 %; PGE_2 + EP2 + EP4 antagonist = 77 ± 10 % (*Figure 4.4b*) This shows that the EP4 receptor antagonist is inhibiting the rise in cAMP seen in response to a 10 min stimulation with PGE_2. The combination of EP2 and EP4 receptors is also inhibiting this. These 2 inhibitory actions are significant ($p < 0.01$) when compared to PGE_2 treatment with no antagonist. Addition of an EP2 antagonist alone appears to cause a stimulation of cAMP production which is significant when compared to PGE_2 treatment with no antagonist ($p < 0.05$).

4.3.2 MAPK results

4.3.2.1 PGE_2 and $PGF_{2\alpha}$ do no affect ERK1/2 phosphorylation in fetal membranes

The results of a time course experiment involving treatment of amnion and chorio-decidua with PGE_2 and $PGF_{2\alpha}$ and PD98059 and U73122 inhibitors and measuring ERK1/2 phosphorylation is shown in *Figure 4.5*. The graphs show ERK1 phosphorylation levels relative to a control that had not been treated with PG. Administration of either PG caused no change to ERK1 phosphorylation, results that were mirrored with ERK2 phosphorylation. Further to this, neither PD98059 nor U73122 inhibitors caused a change to ERK phosphorylation.

4.3.2.2 PGE_2 and $PGF_{2\alpha}$ increase ERK1/2 phosphorylation in JEG3 cells

Treating JEG3 cells with both PGE_2 and $PGF_{2\alpha}$ for 10 min caused an increase in ERK phosphorylation (*Figure 4.6*). ERK1/2 phosphorylation occurred in a time-dependent fashion with a slight stimulation after 5 min PGE_2 treatment, a significant ($p < 0.05$) stimulation after 10 min, and further stimulation after 15 min. With $PGF_{2\alpha}$ treatment, ERK phosphorylation increased significantly after 10 min ($p < 0.05$) and further after 15 min. Pre-treatment with PD98059 inhibited this stimulation after both treatments and at all time points, however this was only significant after 10 min with both PGs ($p < 0.05$ compared to control after 10 min). U73122 appeared to partially inhibit the PGE_2

response after 10 and 15 min and the $PGF_{2\alpha}$ response after 15 min, although these results were not significant.

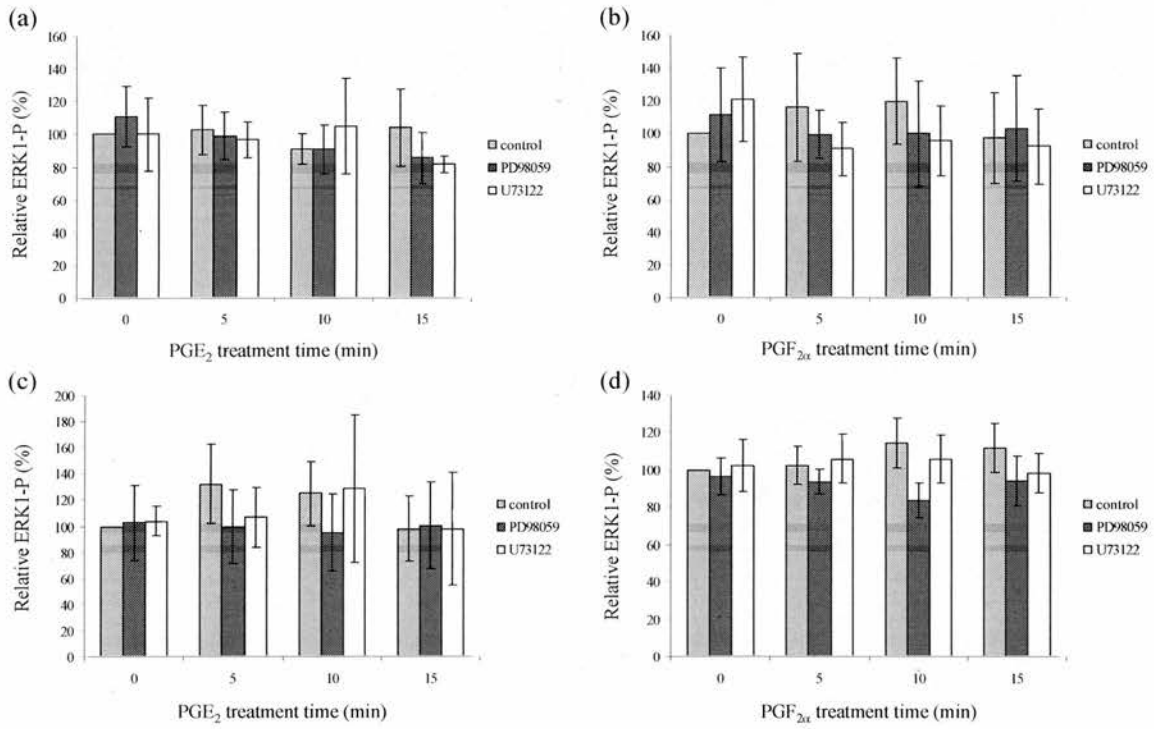


Figure 4.5: Relative ERK1 phosphorylation levels, as determined by densitometric analysis of Western blots, on treatment with PGE_2 ((a) and (c)) and $PGF_{2\alpha}$ ((b) and (d)) in the amnion ((a) and (b)) and chorio-decidua ((c) and (d)). The effects of pre-treatment with inhibitors PD98059 and U73122 are shown. n=4 patients, with each treatment replicated 3 times.

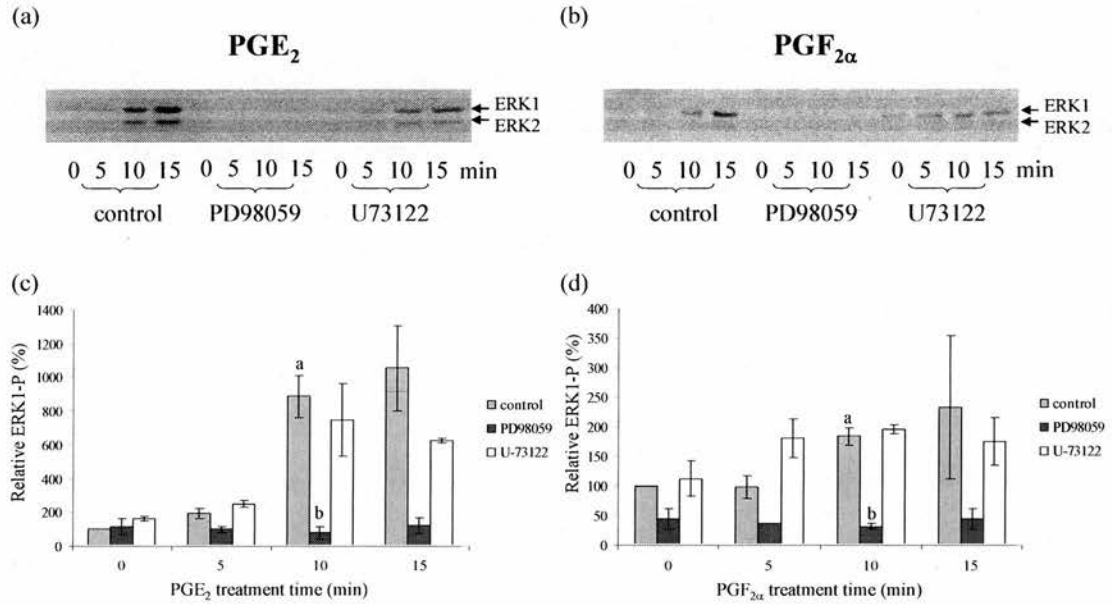


Figure 4.6: Western blot of ERK1/2 in JEG3 cells upon time course treatment with 100 nM PGE_2 (a) and 100 nM $PGF_{2\alpha}$ (b). Effects of treatment of PD98059 and U73122 are shown. Densitometric analysis of the Western blot for relative ERK1 phosphorylation is shown in (c) for PGE_2 and (d) for $PGF_{2\alpha}$. (a = $p < 0.05$ compared to time 0 values and b = $p < 0.05$ compared to un-inhibited samples). n=3.

4.3.2.3 EP4 and FP antagonists inhibit PG induced ERK1/2 phosphorylation in JEG3 cells

Pre-treatment with an EP4 or an FP receptor antagonist inhibited the $PGE_2/PGF_{2\alpha}$ increase in ERK phosphorylation (*Figure 4.7*). Relative ERK1 phosphorylation levels were as follows: for PGE_2 treatment - no antagonist – 290 ± 15 %; EP2 antagonist – 430 ± 69 %; EP4 antagonist – 203 ± 10 %; EP2+EP4 antagonists – 204 ± 7 %; for $PGF_{2\alpha}$ treatment – no antagonist – 264 ± 0.5 %; FP antagonist – 141 ± 13 %. These changes were mirrored in the ERK2 phosphorylation levels. EP2 receptor antagonist treatment increased ERK phosphorylation after 10 min PGE_2 treatment compared to a control with no antagonist. An EP4 receptor antagonist, and a combination of EP2 and EP4 antagonists inhibited the response to PGE_2 significantly ($p < 0.05$) compared to a control with no antagonist. FP antagonist significantly inhibited the $PGF_{2\alpha}$ response after 10 min ($p < 0.05$ compared to $PGF_{2\alpha}$ control with no antagonist).

4.3.2.4 An EGFR kinase inhibitor does not affect PG stimulation of ERK1/2 phosphorylation in JEG3 cells

Pretreatment with AG1478 had no significant effect on the PGE_2 or $PGF_{2\alpha}$ stimulation of ERK1/2 phosphorylation. The graph in *Figure 4.8* shows these results for ERK1 phosphorylation. The same pattern was observed for ERK2 phosphorylation.

4.3.3 Inositol phosphate release by JEG3 cells is not affected by addition of $PGF_{2\alpha}$

No changes in release of total inositol phosphate from JEG3 cells occurred upon treatment with either of the administered doses of $PGF_{2\alpha}$ (*Figure 4.9*).

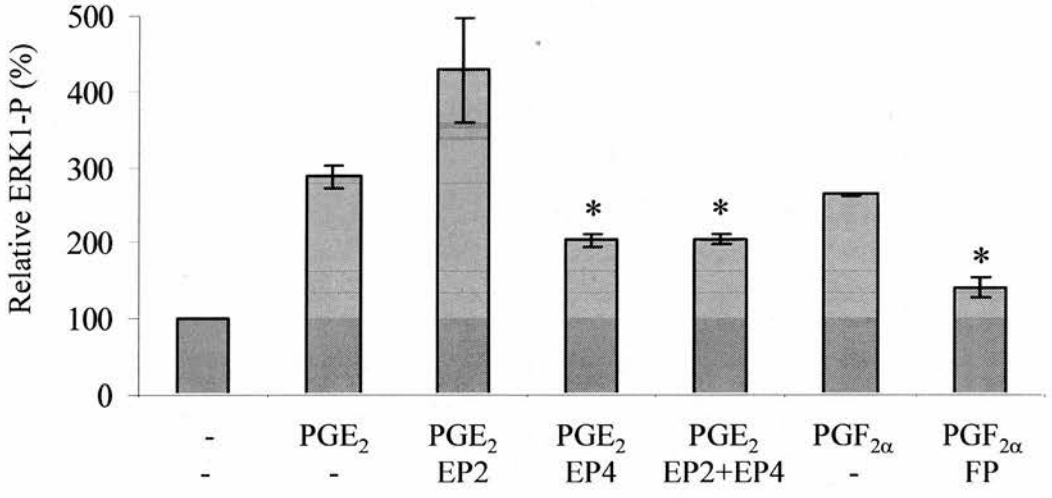


*Figure 4.7: Effect of EP2, EP4 and FP receptor antagonists on ERK1 phosphorylation in JEG3 cells. Treatments consisted of 100 nM PGE_2 and 100 nM $PGF_{2\alpha}$ for 10 minutes with or without antagonists. * denotes a significance value of $p < 0.05$ compared to the samples with no antagonist. n=2 experiments with 2 repeats for each treatment.*

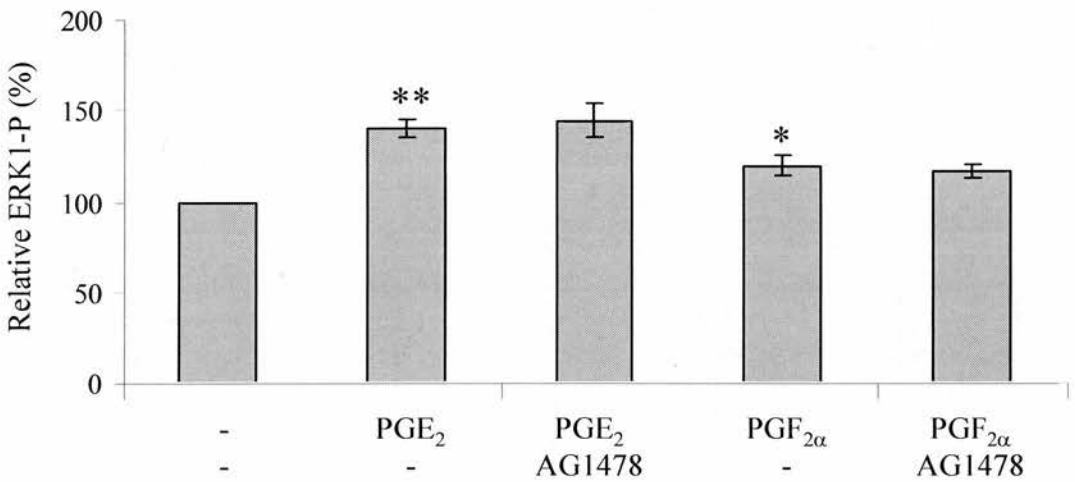


*Figure 4.8: Relative ERK1 phosphorylation, as determined by densitometric analysis of Western blotting, showing the effect of AG1478, an EGFR inhibitor. Treatments consisted of 100 nM PGE_2 and 100 nM $PGF_{2\alpha}$ for 10 minutes with or without AG1478. ** denotes a significance value of $p < 0.01$ and * = $p < 0.05$ compared to untreated cells. AG1478 had no significant effect on the increase in ERK1 phosphorylation caused by PGE_2 and $PGF_{2\alpha}$. n=2, with treatments repeated twice.*

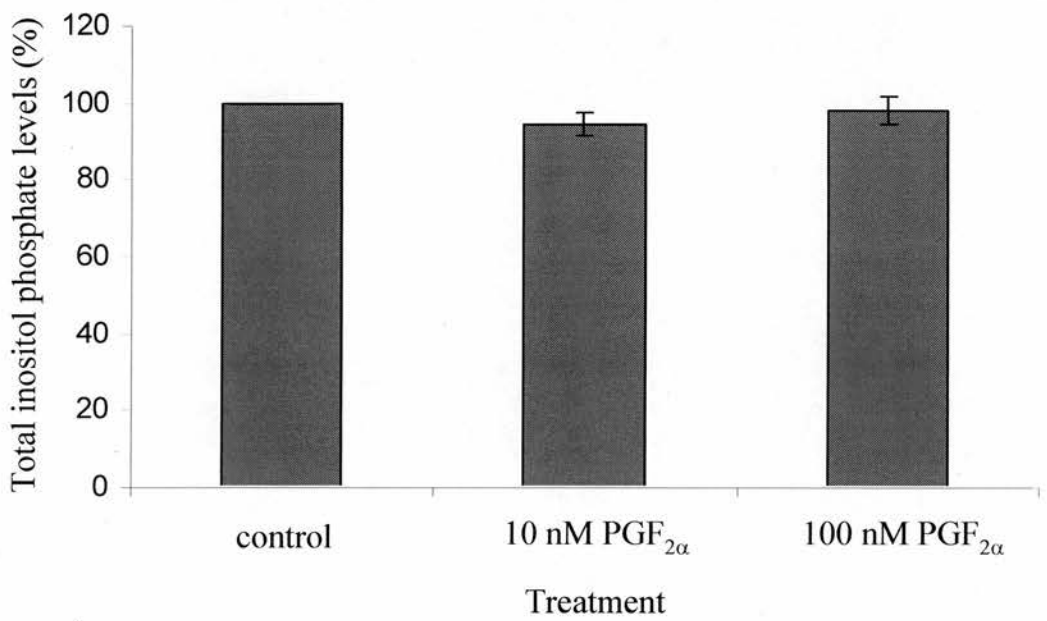


Figure 4.9: Relative total inositol phosphate release in JEG3 cells treated with 10 nM or 100 nM $PGF_{2\alpha}$ for 1 hr at 37 °C. n=2 independent experiments, with treatments repeated in triplicate.

4.4 Conclusions

During parturition, PGE_2 and $PGF_{2\alpha}$ exert many different effects on human uterine and gestational tissue such as stimulating myometrial contractions, ripening of the cervix and rupture of the fetal membranes. These actions are mediated via specific GPCRs. In the previous chapter, it was shown that receptors for these ligands, EP2, EP4 and FP are expressed in human amnion and chorio-decidua and in JEG3 cells. The results in this chapter show that PGE_2 and $PGF_{2\alpha}$ can cause phosphorylation of ERK1/2 via a MEK dependent pathway mediated through EP4 and FP receptors in JEG3 cells. This pathway is not PLCβ dependent, as shown by lack of inhibition with U73122. It is also clear that PGE_2 is acting via the EP2 receptor in human amnion, and the EP4 receptor in JEG3 cells to provide signalling via the cAMP pathway. These results and are summarised in the diagram in *Figure 4.10* which shows a model of the signalling cascades activated by prostaglandins in the JEG3 cells.

Measuring cAMP generation in response to PGE_2 assessed functional signalling of the EP2 and EP4 receptors, which are both coupled to stimulatory G-proteins. Through the use of selective receptor antagonists, it was shown that PGE_2 can activate the cAMP/PKA pathway via the EP2 receptor in human amnion, and via the EP4 receptor in JEG3 cells. The pattern of response in both the JEG3 cells and the amnion tissue was with a peak after 10 minutes, and a slight reduction compared to this after 15 min. The time scale was extended in the JEG3 cells experiments and this pattern was similar after a further 15 min. This trend could be due to cAMP degradation within the cell, or cAMP release from the cells during this time course. No significant cAMP response to PGE_2 was observed in the chorio-decidua samples, although a small elevation was seen in a similar pattern and time-scale to that seen in the amnion and JEG3 cells. Due to the number of cell types in these samples, a positive response in one cell type may be being masked by another. For example, while the trophoblast cells contain the EP2 and EP4 receptors which are couple to a stimulatory G protein, it is possible that these cells, and also the cells in the reticular layer contain an isoform of the EP3 receptor which is

coupled to an inhibitory G protein. Thus, while in some cell types, PGE_2 may be elevating cAMP, in others, it may be inhibiting cAMP production, these effects cancelling each other out, showing no overall changes. The JEG3 trophoblast cells did show a response, so it is possible that the trophoblast cells of the chorion, which have been shown to contain the EP2 and EP4 receptors, would produce a cAMP response to PGE_2 treatment.

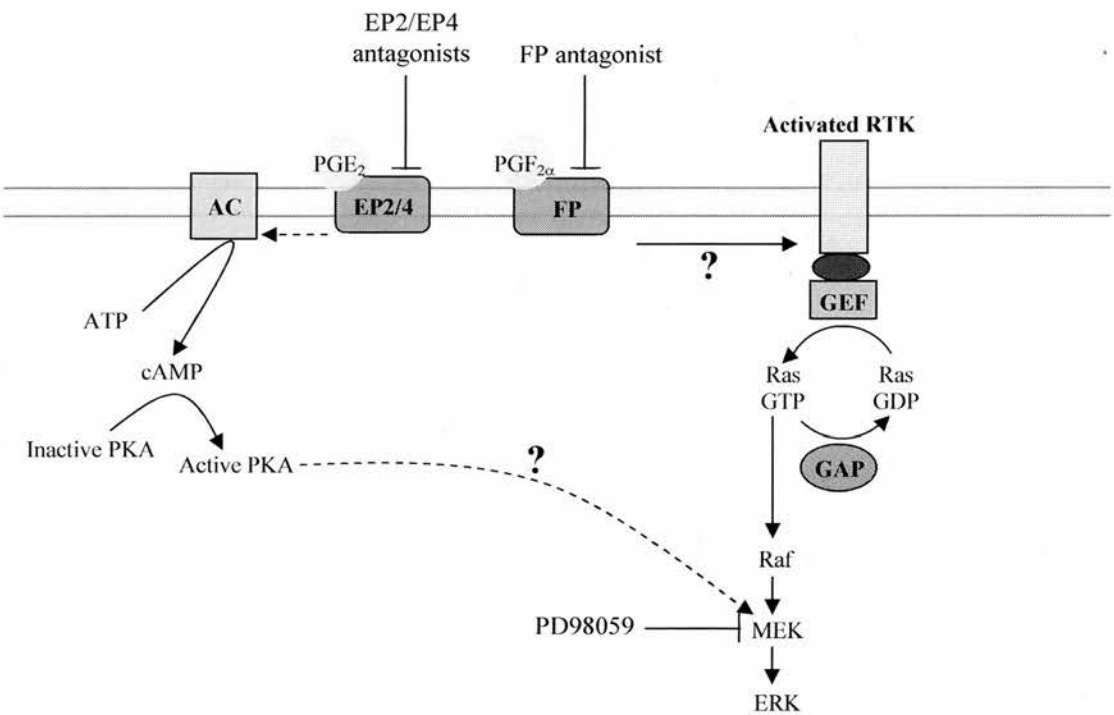


Figure 4.10: Proposed mechanism of prostaglandin intracellular signalling in the JEG3 cells. AC; adenyl cyclase, PKA; protein kinase A, ERK; extracellular signal regulated kinase, MEK; ERK kinase, GTP; guanosine triphosphate, GDP; guanosine diphosphate, GEF; guanine exchange factor, GAP; GTPase activating protein.

In the JEG3 cells, the small but significant increase in cAMP generation and the slight (though not significant) increase in ERK phosphorylation after treatment with PGE_2 and an EP2 antagonist was surprising. A possible explanation is that, in the presence of the EP2 antagonist, there is more PGE_2 available for stimulating the EP4 receptor, which has been shown to be functional in cAMP and ERK signalling in these cells. This is supported by the fact that a combination of EP2 and EP4 antagonists inhibits the cAMP generation and ERK phosphorylation to the same level as that seen with an EP4 antagonist alone. It is also possible that the EP2 antagonist is having agonist effects, possibly on the EP4 receptor, or the EP1 or EP3 receptors. To fully understand this system of PGE_2 signalling, analysis of EP1 and EP3 receptors would also have to be carried out.

It is not known what role this elevation of cAMP in response to PGE_2 specifically does in human fetal membranes. It has been shown that PGE_2 induced elevations of cAMP in pregnant rat uterine and cervical tissue can regulate MMP levels (Lyons et al 2002). In this study, the authors found that in uterine and cervical tissues, PGE_2 up-regulated cAMP significantly in term and preterm pregnant tissue, but not in non-pregnant tissue. MMP-2 and MMP-9 levels were also elevated in response to PGE_2 in the same circumstances (except uterine MMP-9 levels were unchanged in term tissue). As cAMP has been shown to induce MMP-2 mRNA transcription (Hasan & Nakajima 1999), the authors suggested that the PGE_2 effect on MMP levels is being mediated via the cAMP pathway in these rat tissues. It has also been suggested that the PGE_2 induced elevation in cAMP levels play a role in functional progesterone withdrawal in myometrial cells at labour by increasing the progesterone receptor (PR)-A/PR-B expression ratio. (Madsen et al 2004a). PGE_2 and $PGF_{2\alpha}$ both increased progesterone receptor expression. Addition of a cAMP analogue, 8-Br-cAMP activated the PKA pathway, as well as increasing PR-A and PR-B expression, without altering the PR-A/PR-B ratio. Activation of PKC however, increased expression of only PR-A, and not PR-B, thus increasing the PR-A/PR-B ratio. This shows that in the myometrium, a PGE_2 induced cAMP elevation

does not facilitate functional progesterone withdrawal, however, $PGF_{2\alpha}$ activation of the PKC does.

The MAPK pathways are a key signalling mechanism in cells and are a potent regulator of cell growth, differentiation and development (Lewis et al 1998). MAPK signalling can be activated by RTKs, GPCRs via activation of small G-proteins such as Ras and Raf, or by PKA via activation of Rap1 (Stork 2003).

From the results in this chapter, activation of the ERK pathway by both PGE_2 and $PGF_{2\alpha}$ in JEG3 cells indicates functional receptor signalling in choriocarcinoma trophoblast cells. Phosphorylation of ERK1/2 is inhibited by pre-treatment of JEG3 cells with the MEK inhibitor, PD98059. However, no decrease in PGE_2 or $PGF_{2\alpha}$- induced phosphorylation of ERK1/2 was observed with U73122, indicating that ERK1/2 phosphorylation is not mediated via PLCβ. As with the PGE_2 induced cAMP elevation, the ERK1/2 phosphorylation was shown to be mediated via the EP4 receptor. However, addition of this antagonist, or a combination of the EP2 and EP4 antagonists, only partially inhibited the PG mediated ERK1/2 phosphorylation. This suggests that other receptors, such as EP1 and EP3 may also be functionally active in these cells and mediating PG induced ERK1/2 phosphorylation.

MAPK/ERK phosphorylation is usually a consequence of activation of tyrosine kinase receptors (RTKs). Activation of the MAPK pathway via the FP receptor, which is $G_{\alpha q}$-coupled, can be explained by activation of PLCβ and PKC and subsequent phosphorylation of Raf (MAPK kinase kinase). Data has suggested that GPCRs, in addition to activating G-proteins, also activate MAPK kinase cascades (Faure et al 1994, Lewis et al 1998). However, the way in which signals are transduced from GPCRs to the MAPK cascade has been a matter of debate for years. Recent advances suggest that RTK transactivation is an important pathway that links these GPCRs and the MAPK pathway (Wetzker & Bohmer 2003). This was first seen when several GPCR agonists were

shown to have an effect on the EGF receptor in fibroblasts (Daub et al 1996). This link between RTKs and GPCRS has been seen in at least 3 RTKs: EGF, PDGF and IGF-1 all become tyrosine phosphorylated after GPCR activation (Luttrell et al 1999).

There are a few models for the transactivation of RTKs by GPCR, one of these being a 'triple-membrane-passing-signalling mechanism' (TMPS). This was first proposed when it was found that GPCR-dependent stimulation of EGFR involved stimulation of the matrix metalloproteinases, which in turn induce the extracellular release of heparin binding-EGF (HB-EGF) from its latent membrane spanning precursor form in the plasma membrane (Prenzel et al 1999). Similar findings have been shown by others since (Fujiyama et al 2001, Pierce et al 2001). A diagram of this model is shown in *Figure 4.11a*. Different mediators of the Scr-family kinases are involved, as are Ca^{2+} and PKC. Additional pathways of EGFR transactivation that do not involve metalloproteinase-mediated HB-EGF release have also been identified. One mechanism involves the GPCR-triggered recruitment of RTK in a complex with cytoplasmic TKs, for example Src and Pyk2 (*Figure 4.11b*). Both of these kinases interact with the EGFR (Biscardi et al 1999, Keely et al 2000) and Src has also been shown to directly phosphorylate and activate the EGFR (Biscardi et al 1999). Another model suggests that GPCR activation may lead to the production of H_2O_2 through the activation of NAPDH oxidases (*Figure 4.11c*). Protein tyrosine phosphatases (PTPs), which negatively regulate RTK activity are very sensitive to oxidation, are transiently inactivated (Cys-SH oxidised to Cys-SOH) by H_2O_2, thus inhibiting the deactivation of RTK signalling (Rhee et al 2000, Wetzker & Bohmer 2003).

Prostaglandin transactivation of RTKs has been observed. In human endometrial adenocarcinomas, $PGF_{2\alpha}$ binding to the FP receptor has been shown to induce transactivation of EGFR (Sales et al 2004b), as has PGE_2 via the EP2 and EP4 receptors (Sales et al 2004a). Thromboxane can activate the ERK pathway through its TP receptor, and this involves transactivation of EGF (Miggin & Kinsella 2001).

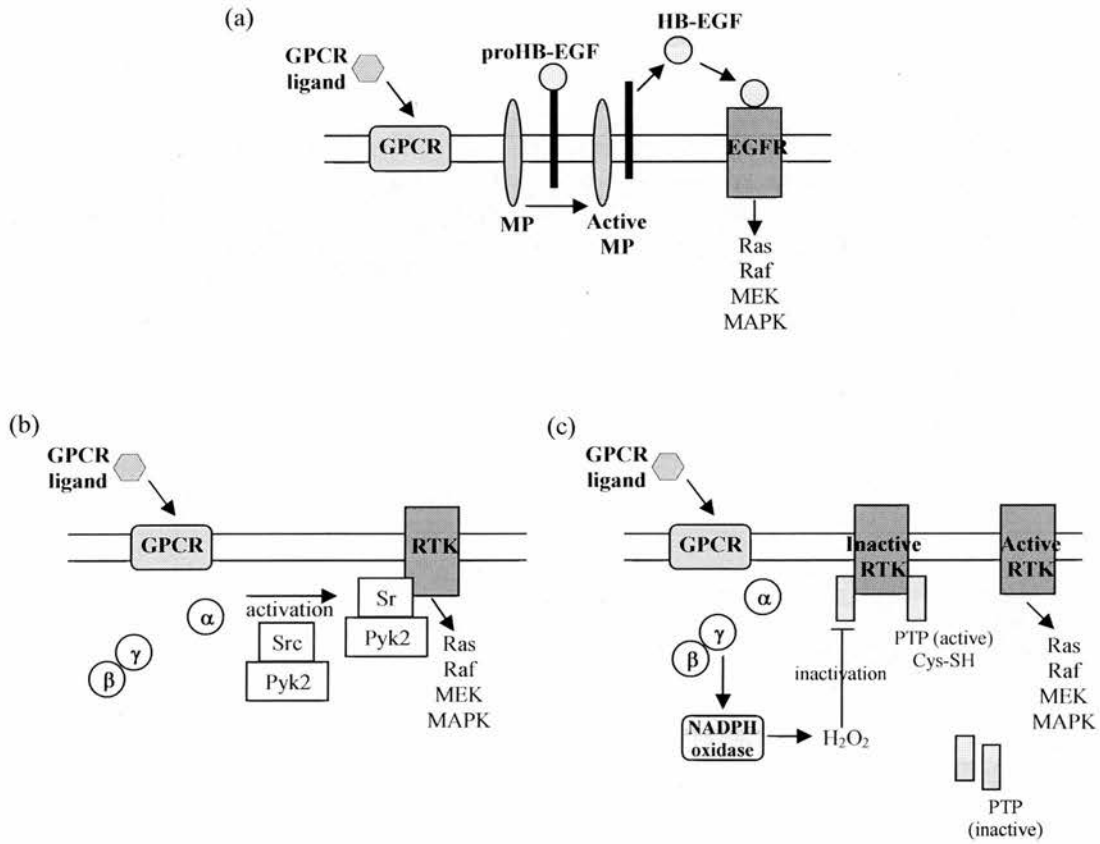


Figure 4.11: Possible mechanisms for transactivation of receptor tyrosine kinases (RTKs) by G-protein coupled receptors (GPCRs). (a) the triple-membrane-passing-signalling pathway (TMPS); (b) a mechanism involving the recruitment of cytoplasmic tyrosine kinases; and (c) a route through the activation of NADPH oxidases and the subsequent oxidation and inactivation of protein-tyrosine phosphatases (PTPs). Figure adapted from Wetzker and Bohmer 2003.

The JEG3 cells express both plasma membrane bound EGFR (Cao et al 1994, Fulop et al 2001) and nuclear EGFR (Cao et al 1995). To ascertain whether the observed stimulation of the ERK pathway with both PGE_2 and $PGF_{2\alpha}$ is linked to the EGFR, cells were preincubated with an inhibitor of EGFR kinase, AG1478, prior to stimulation with $PGE_2/PGF_{2\alpha}$. No inhibition of the $PGE_2/PGF_{2\alpha}$ induced stimulation of ERK1/2 phosphorylation was observed upon addition of AG1478. It may be that another RTK, such as PDGF is transactivated by the prostaglandin receptors, to activate the ERK pathway. It is also possible that GPCR-dependent stimulation of the ERK/MAPK cascade does not involve RTKs. For example, non-receptor tyrosine kinases, such as focal adhesion kinase (FAK) may be involved. FAK is activated by cell binding to ECM components, and by several agonists acting on GPCRs, such as vasopressin, bradykinin and endothelin (Igishi et al 1999, Salazar et al 2003). FAK recruits other non-receptor tyrosine kinases and can activate the ERK/MAPK cascade. The FP receptor has also been shown to be coupled to Rho, a small G-protein which is upstream of FAK phosphorylation (Pierce et al 1999). Further experiments using tyrosine kinase inhibitors of other RTKs, or small G-proteins and adaptor molecules would improve our understanding of the intracellular signalling events involved in prostaglandin stimulation of their receptors in JEG3 cells.

The role of prostaglandin induced MAPK pathway stimulation in human uterine and gestational tissues is unclear. G-proteins have been shown to play a pivotal role in smooth muscle activation and relaxation by coupling cell membrane receptors to effector enzymes and ion channels. Clearly, the activation of various transcription factors via this pathway may play a role in regulation of expression of mediators involved in pregnancy. Activation of other proteins by phosphorylation may also play a role.

No evidence of levels of inositol triphosphate changes are evident upon treatment of JEG3 cells with $PGF_{2\alpha}$ indicating that this ligand does not activate the inositol triphosphate signalling pathway in these cells. Further evidence for this pathway not being activated comes from a lack of inhibition of PLCβ by U73122 in ERK1/2

phosphorylation. If the IP3 pathway had been stimulated, it would be expected that PKC would have been activated and henceforth the MAPK phosphorylation cascade. If this was the case, ERK1/2 phosphorylation would be expected to be inhibited by inhibitors of this pathway. This suggests that ERK1/2 phosphorylation via $PGF_{2\alpha}$ activation of the FP receptor is dependent on transactivation of RTK.

Taken together, the results from this chapter show that the EP2 receptor is functionally active in human amnion and that in this tissue, PGE_2 stimulation of cAMP occurs via this receptor. In the JEG3 cells, PGE_2 is acting via the EP4 receptor to elevate cAMP production, and via EP4 and FP to mediate PGE_2/ $PGF_{2\alpha}$ stimulated phosphorylation of ERK1/2. The chorio-decidua showed no response to PGs with regards to either of these pathways which were activated in JEG3 cells. Although JEG3 cells are derived from human trophoblast cells, they are an immortalised cell line, and exhibit different receptor-ligand interactions. The chorio-decidua explants used contain a variety of cell types, giving rise to different paracrine signalling mechanisms. Unless specific chorion trophoblast cells were isolated for this experiment it will not be known whether the same effect occurs in this human tissue as in the JEG3 cells.

It has been shown that MMP expression and activation can be increased by PGE_2 and $PGF_{2\alpha}$ (McLaren et al 2000b, Ulug et al 2001) and this regulation may be occurring via intracellular signalling pathways such as the MAPK cascade, or the cAMP/PKA pathway. It has been suggested that ERK1/2 regulates MMP-2 production in JEG3 cell culture (Andrassy et al 2004). These relationships will be further examined in the next chapter.

Chapter 5: MMP Production and Regulation in Human Fetal Membranes and Placenta

5.1 Introduction and Aims

Extracellular matrix (ECM) remodelling is found in many processes during human parturition at term and preterm. These include cervical ripening, fetal membrane rupture, and placental detachment from the maternal uterus (Bryant-Greenwood & Yamamoto 1995, Rajabi et al 1988, Tsatas et al 1999). Matrix metalloproteinases (MMPs) are the main mediators of ECM degradation, particularly the gelatinases, MMP-2 and MMP-9 have been implicated in parturition (Riley et al 1999a, Stygar et al 2002, Vadillo-Ortega et al 1995, Xu et al 2002). The activity of MMPs is regulated by their physiological inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), inhibition occurring by the formation of a 1:1 MMP:TIMP complex.

It has been found that levels of certain MMPs are present and elevated in uterine and gestational tissues at labour. MMP-9 production in the rat amnion is induced prior to labour (Lei et al 1995). Similar changes are seen in the human. MMP-9 production is elevated in amnion, chorion, decidua and placenta (Goldman et al 2003, McLaren et al 2000a, Tsatas et al 1999, Uchide et al 2000, Xu et al 2002) with the activity increasing in the amnion at the onset of labour (Goldman et al 2003, Maymon et al 2000a, McLaren et al 2000a, Vadillo-Ortega et al 1995). MMP-2 production is also elevated in amnion, chorion, decidua and placenta (Goldman et al 2003, McLaren et al 2000a, Xu et al 2002). Activity of MMP-2 is increased in decidua with labour, with no changes seen in amnion, chorion or placental samples (Goldman et al 2003, Xu et al 2002). MMP-2 and MMP-9 production both increase in human cervical tissue at term, implicating both these enzymes in the process of cervical ripening (Stygar et al 2002). Decreases in levels of TIMPs and an increase in the balance of the MMP:TIMP ratio have also been implicated in labour (Riley et al 1999b, Vadillo-Ortega et al 1996).

Prostaglandins and cytokines may be among the regulatory factors responsible for the increased levels of MMP expression and activity in the fetal membranes associated with rupture, and may also be involved in cervical ripening. In fact MMP-9 secretion from

human cervical fibroblasts (Sato et al 1996), trophoblasts (Shimonovitz et al 1996) and endometrial stromal cells (Huang et al 1998) are stimulated by hormones and cytokines. Intrauterine infection has been associated with higher MMP-9 concentrations in amniotic fluid, whether or not rupture occurred (Athayde et al 1998, Draper et al 1995, Vadillo-Ortega et al 2002). MMP-9 is stimulated by IL-1β and TNF-α in human amnion but not chorion (Arechavaleta-Velasco et al 2002). In the rhesus monkey, bacterial and IL-1β induced preterm labour and spontaneous labour are preceded and accompanied by progressive increases in amniotic fluid MMP-9 levels (Vadillo-Ortega et al 2002). Thus, amniotic fluid MMP-9 may be a good clinical marker for onset of both preterm and term labour. The role of MMP-9 as a clinical marker for preterm labour and intra-amniotic infection has been investigated and was found to be a potentially valuable tool (Locksmith et al 1999). Cytokine effects on human myometrial smooth muscle MMP production have also been studied. IL-1β and TNF-α both induced MMP-9 production, though 17β-oestradiol and progesterone did not (Roh et al 2000). Progesterone did not decrease the cytokine mediated stimulation of MMP-9 either, despite it having been shown to be a physiological suppressor of MMP-9 (Roh et al 2000). In human trophoblast cells, MMP-9 is suppressed by progesterone (Shimonovitz et al 1998), which also occurs in rabbit cervical fibroblasts (Imada et al 1997), and progesterone also increases TIMP-2 expression in these cells (Imada et al 1994).

Various prostaglandin effects on MMPs have been reported. PGE_2 has been linked to elevated MMP-9 production by cultured human fetal membranes (McLaren et al 2000b). This PG has also been shown to elevate plasma, uterine and cervical MMP-2 levels in preterm and term pregnant rats, and to induce MMP-9 in preterm plasma and uterus in term pregnant rats (Lyons et al 2002). $PGF_{2\alpha}$ elevates MMP-2 and MMP-9 production, and MMP-2 activation and decreases TIMP-1 levels in human term decidua, with no effect on these proteins in fetal membrane samples (Ulug et al 2001). Prostacyclin has been shown to be concerned with inhibiting MMP-9 production in human cultured mesangial cells (Kitahara et al 2001), although no role has been found in tissues involved in labour. COX-2 promotes the release of MMP-2 and MMP-9 in fetal rat

hepatocytes (Callejas et al 2001), but again there is no evidence for involvement of this process in labour.

In this chapter, the effect of prostaglandins, PGE_2 and $PGF_{2\alpha}$ on the production and activation of MMP-2, MMP-9 and TIMPs in cultured human fetal membranes and placenta, and the JEG3 choriocarcinoma cell line will be examined. The effect of a pro-inflammatory cytokine, IL-6 on MMP-2 in the JEG3 cell-line will also be investigated.

5.2 Methods

5.2.1 Effect of PGE_2 and $PGF_{2\alpha}$ on MMP-2 and MMP-9 Production and Activation in Human Fetal Membranes

Amnion and chorio-decidua were collected and cultured as in section 2.2.1. Treatments added to the 1 ml culture medium for a 24 hr incubation were 100 nM, 10 μM and 100 μM PGE_2 and $PGF_{2\alpha}$ and a control with no treatment. Background samples were also taken which consisted of 1 ml media without tissue or treatment. Culture medium harvested from these wells was freeze dried and amnion samples were reconstituted in 100 μl water, chorio-decidua samples in 50 μl water. 5 μl samples were mixed with an equal volume of zymography sample application buffer (appendix 1.5.2.1) and zymography carried out. The zymograms were analysed by densitometry and values expressed as a percentage of the control. Reverse zymography was also carried out on the same samples with 7.5 μl sample mixed with 7.5 μl reverse zymography application buffer. n=5 patients, and each treatment was carried out in quadruplicate.

5.2.2 Effect of PGE_2 and $PGF_{2\alpha}$ on TIMP Production in Human Fetal Membranes

Reverse zymography, to detect TIMP activity, was also carried out on all the samples above, with 7.5 μl sample mixed with 7.5 μl sample application buffer loaded into the gels. This allows identification of TIMP-2, an unglycosylated form of TIMP-3, and a

band at the molecular weight of TIMP-1, 4 and a glycosylated version of TIMP-3. To further clarify these bands, Western Blot analysis was carried out. Equal volumes of each of the 4 quadruplicates were pooled (to provide enough volume of sample) and 20 μl sample mixed with 20 μl Western sample application buffer (appendix 1.5.1.3) and loaded into a well. The gels used were 12 % acrylamide. The procedure was carried out as in section 2.7.2.3. The primary antibody incubation time was 2.5 hr. Exposure time was dependent on the blot. The primary antibodies for TIMP-1, 2, 3 and 4 were all human anti-rabbit monoclonal antibodies and used at a dilution of 1:1000 from the stock. The secondary antibody was a donkey anti-rabbit. Reverse zymograms and Western Blots were analysed by densitometry using Quantity One.

5.2.3 Indomethacin Effect on PGE_2 in Amnion and Chorio-decidua

Samples of amnion, chorio-decidua and placenta were collected and divided into 3 categories: no indomethacin treatment, indomethacin pre-treatment, and indomethacin pre-treatment and treatment. The first group were treated as previously, with a 2 hr incubation in steeping buffer. The second and third groups had additionally 10 μM indomethacin added at this stage. The third group had 10 μM indomethacin added to the wells, along with the prostaglandins for the 24 hr incubation. The control samples with no prostaglandin also received indomethacin. After the 24 hr incubation, medium was treated 1:1 with methyloximating solution and a PGE_2 assay carried out. The tissue was also removed and fixed (section 2.1.2) and H and E staining carried out (section 2.4.2.4). In this experiment, n=3 patients.

5.2.4 Effect of PGE_2 and $PGF_{2\alpha}$ on MMP-2 and MMP-9 with Indomethacin Pre-treatment

Amnion, chorio-decidua and placenta were collected as previously described. They were treated in steeping buffer containing 10 μM indomethacin prior to being cut and explants placed in wells. They were then treated with either 100 nM PGE_2 or100 nM $PGF_{2\alpha}$ or not treated as a control, with 6 wells of a 24 well plate for each treatment. Treatments

were left for 8 or 24 hr, upon which medium was collected (500 μl each combined to 1 ml from 2 duplicate wells, leaving 3 samples of 1 ml for each treatment). Medium was frozen at – 70 °C before being freeze dried, and amnion samples reconstituted in 50 μl, chorio-decidua and placenta samples in 100 μl. 7 μl of each sample, combined with 7 μl sample application buffer was loaded into gels and zymography performed. n=4 patients with each treatment being carried out in triplicate.

The same experiment was also performed using JEG3 cells. These were plated out as in section 2.3 and left for 2 days to stick down and reach confluence. At this stage, medium was aspirated and replaced with serum free medium with 10 μM indomethacin for 24 hr. This was aspirated and treatments (control, 100 nM PGE_2 or $PGF_{2\alpha}$ and also 0.5 nM IL-6) added for 2, 8, 12 and 24 hr. 1 ml of medium was collected, freeze dried and reconstituted in 50 μl water. Zymography was performed on these samples, as above.

5.2.5 Effect of PGE_2 and $PGF_{2\alpha}$ on TIMP Production with Indomethacin Pre-treatment

Using the samples from section 5.2.4, TIMP production was also analysed. The triplicates (same treatment) of the samples from each patient were pooled and dialysed before being freeze dried and run on a gel and Western blotted for TIMPs 1-4, as in section 5.2.2. The samples were also analysed by reverse zymography.

5.2.6 Cellular MMP-2 and MMP-9 Content in JEG3 Cells

JEG3 cells were also homogenised after aspiration of the medium to examine intracellular levels of MMP. Here, cells were plated out in 75 cm^3 flasks with 25 ml of cells at 2 x 10^5 cells/ml, left to reach confluence, then left with serum free medium for 24 hr. The medium was then removed, and the cells trypsinised. 5 ml medium with serum was then added and the cells spun down at 10,000 g for 5 min. The medium was then aspirated from this and the pellet frozen at -20 °C. They were then resuspended in 0.5 ml homogenisation buffer (appendix 1.5.1.1) on ice, and homogenised using the

Polytron on high power for 30 s. They were then centrifuged at 10, 000 g at 4 °C for 15 min. A Western blot was then run, using 20 µl of the homogenised sample and immunoblotted for MMP-2 and MMP-9. These antibodies are both human anti-mouse monoclonal antibodies, used at a dilution of 1:1000 from the stock and detected with sheep anti-mouse secondary antibody. The samples were also analysed by zymography. For this experiment, n=10 individual flasks of cells.

5.2.7 Effect of PGE_2 and $PGF_{2\alpha}$ on MMP-2 and MMP-9 mRNA

Using the same samples as above, at the time when the medium was aspirated, tissue and cells were treated with tri-reagent and RNA extracted (section 2.5.1-2.5.4). The samples were then analysed using the Lightcycler for changes in MMP-2 and MMP-9 mRNA levels (see section 2.5.5 for use of the Lightcycler). G-6-PDH was used as the control gene for both MMP-2 and MMP-9 quantification reactions. The sequences for the primers used are shown in *Table 5.1.* Annealing temperature and $MgCl_2$ concentration were optimised for MMP-2 and MMP-9 and are shown in *Table 5.2.*

Control samples were tested at these conditions to check the standard curve was linear for the MMP and G-6-PDH genes. Then the melt curve was analysed to find the best temperature to make a reading each cycle. These were found to be 87 °C for MMP-2 and 86 °C for MMP-9. Once these reaction conditions were optimised, samples were all run on the lightcycler, in duplicate, to quantify MMP-2 and MMP-9 mRNA.

Product	Primer	Sequence 5' – 3'	Product size
G-6-PDH	forward	CGG AAA CGG TCG TAC ACT TC	210 bp
	reverse	CCG ACT GAT GGA AGG CAT C	
MMP-2	forward	ATG ACA GCT GCA CCA CTG AG	174 bp
	reverse	ATT TGT TGC CCA GGA AAG TG	
MMP-9	forward	TTG ACA GCG ACA AGA AGT GG	179 bp
	reverse	GCC ATT CAC GTC GTC CTT AT	

Table 5.1: Primer sequence and product size for G-6-PDH, MMP-2 and MMP-9 primers.

Primers	Annealing temperature	$MgCl_2$ concentration for lightcycler
MMP-2	61 °C	5 mM
MMP-9	63 °C	4 mM

Table 5.2: Optimised annealing temperature and $MgCl_2$ concentration for use of MMP-2 and MMP-9 primers with the lightcycler.

5.3 Results

5.3.1 Effect of PGE_2 and $PGF_{2\alpha}$ on MMP-2 and MMP-9 Production and Activation in Amnion and Chorio-decidua

Figure 5.1 shows a sample zymogram for each of amnion, chorio-decidua and placenta. The latent forms of MMP-2 and MMP-9 have been detected in all three of the tissue types. It is this latent form of these MMPs that has been analysed. The effects of PGE_2 and $PGF_{2\alpha}$ are shown in 4 graphs in *Figure 5.2*. In the amnion, 1 μM PGE_2 has slightly but significantly increased MMP-2 levels ($p < 0.05$). 100 nM $PGF_{2\alpha}$ has significantly ($p < 0.01$) increased MMP-2 levels slightly and MMP-9 levels to about 250 % of control levels. 1 μM $PGF_{2\alpha}$ has significantly ($p < 0.05$) increased MMP-2 levels, and 10 μM $PGF_{2\alpha}$ has significantly ($p < 0.01$) increased MMP-9 levels to almost 200 % of the control. In the chorio-decidua, 10 μM PGE_2 has slightly ($p < 0.01$) but significantly raised MMP-2 levels, and 100 nM $PGF_{2\alpha}$ has elevated MMP-9 levels slightly ($p < 0.05$).

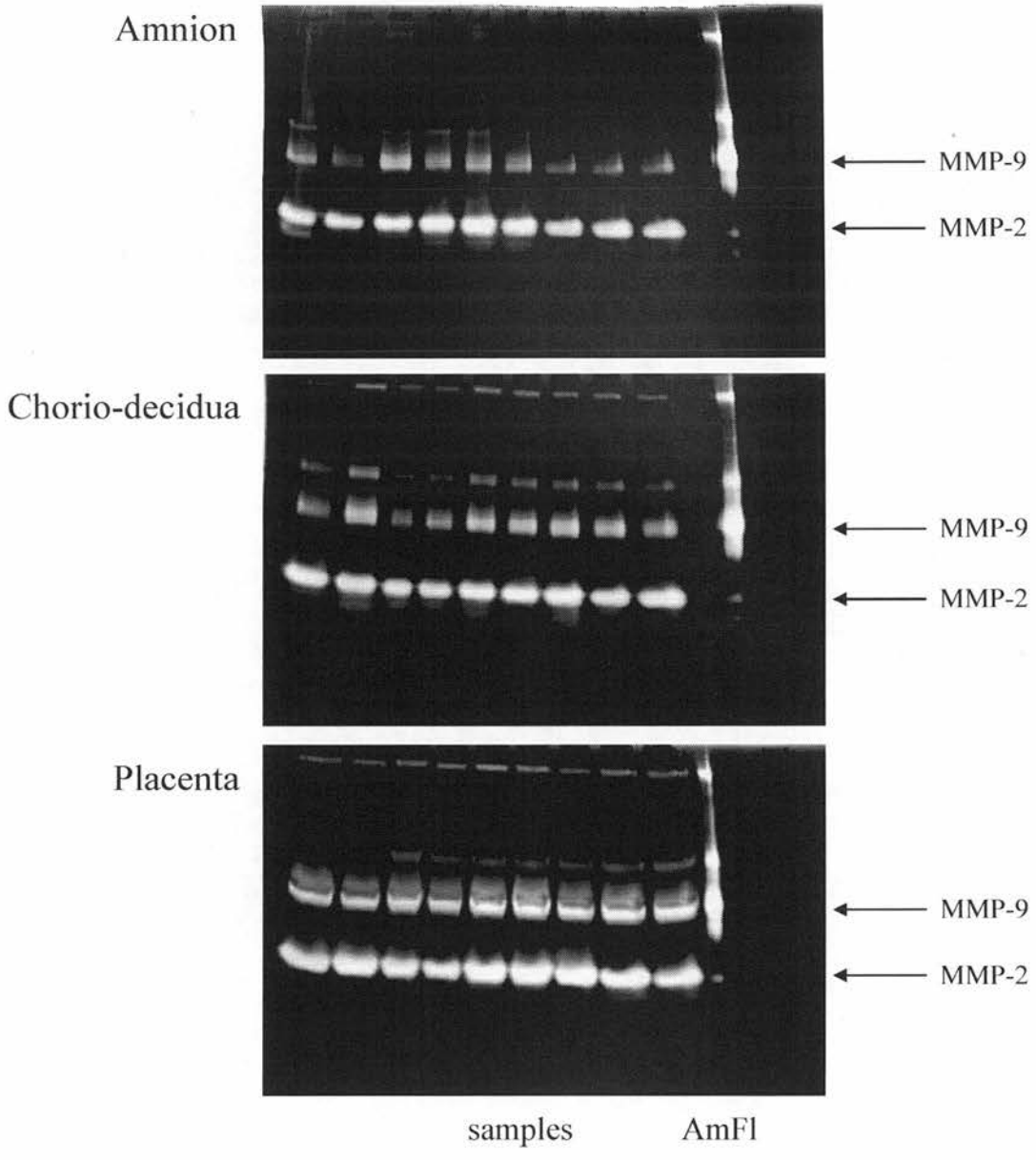


Figure 5.1: Sample zymograms for amnion, chorio-decidua and placenta showing their production of active and latent forms of MMP-2 and MMP-9. The amniotic fluid (AmFl) loaded on the gels acts as a positive control as is has been characterized for MMP-2 and MMP-9.

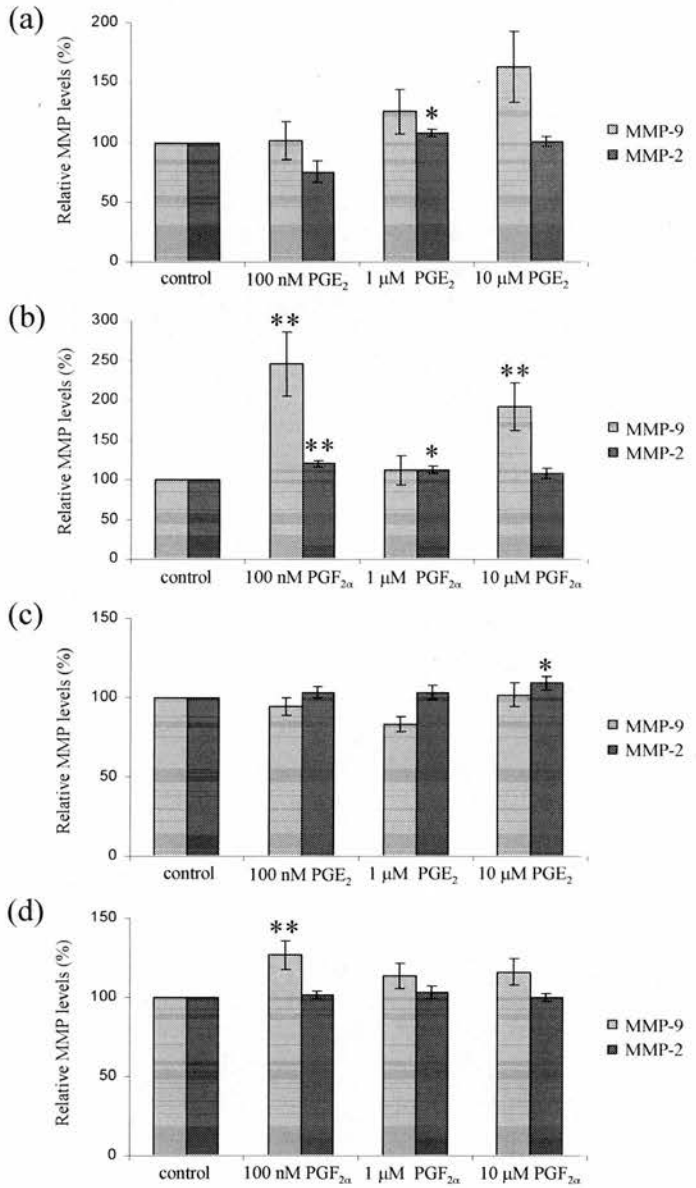


*Figure 5.2: The effects of incubation with 100 nM, 1 µM, or 10 µM PGE_2 (a and c) and $PGF_{2\alpha}$ (b and d) for 24 hr on MMP-2 and MMP-9 production in amnion (a and b) and chorio-decidua (c and d) explants as determined by densitometric analysis of zymograms. * and ** denote significance values of $p < 0.05$ and $p < 0.01$ respectively when compared to control untreated samples. n=5 patients with treatments repeated in quadruplicate.*

5.3.2 Effect of PGE_2 and $PGF_{2\alpha}$ on TIMP Production in Amnion and Chorio-decidua

Figure 5.3 shows a sample reverse zymogram for each of amnion, chorio-decidua and placenta. TIMPs activities at 21, 24 and 27-30 kDa have been detected in all three of the tissue types. Results of reverse zymogram analysis are shown in *Figure 5.4*. It appears that in the amnion, 100 nM PGE_2 has caused over a than 50 % reduction in levels of TIMP with a molecular weight of between 27-30 kDa and 24 kDa. 1 μM PGE_2 has also reduced 27-30 kDa TIMP levels to around 50 % that of controls. 10 μM $PGF_{2\alpha}$ has caused a reduction in a 21 kDa TIMP. No effect of PGs on TIMP levels was shown in the chorio-decidua. The same samples analysed by Western Blot show large standard error of the mean (*Figure 5.5*). No effects were identified in amnion samples, however, these results showed that $PGF_{2\alpha}$ decreased the production of TIMP1 to less than 50 % that of controls.

5.3.3 Indomethacin Effect on PGE_2 in Amnion and Chorio-decidua

It was thought that endogenous protaglandins may be masking effects of the treatments given to tissue. Addition of indomethacin, a dual COX enzyme inhibitor, inhibits endogenous prostaglandin biosynthesis. Levels of PGE_2 were measured before and after treatments with indomethacin to estimate the degree to which it was being inhibited and to compare endogenous levels to doses given as treatments. Endogenous levels in media from untreated tissue in amnion, chorio-decidua and placenta were 1.36 ± 0.66, 0.15 ± 0.04 and 3.51 ± 1.17 ng/ml, respectively (*Figure 5.6a*). This is far lower than the lowest dose of PGE_2 given. which at 100 nM is equivalent to 35 ng/ml. Indomethacin treatment for 2 hours prior to a 24 hr incubation reduced PGE_2 levels to 0.22 ± 0.05 ng/ml in the amnion, 0.10 ± 0.001 ng/ml in the chorio-decidua and 0.77 ± 0.27 ng/ml in the placenta. Levels were further decreased after treatment with indomethacin for the 24 hr incubation (0.20 ± 0.08, 0.08 ± 0.001 and 0.09 ± 0.005 ng/ml in the amnion, chorio-decidua and placenta, respectively). A similar pattern of decreasing PGE_2 concentration with indomethacin treatment was observed with the PGE_2 treatment for 24 hr (*Figure 5.6b*), though to a lesser extent, due to the addition of the PGE_2.

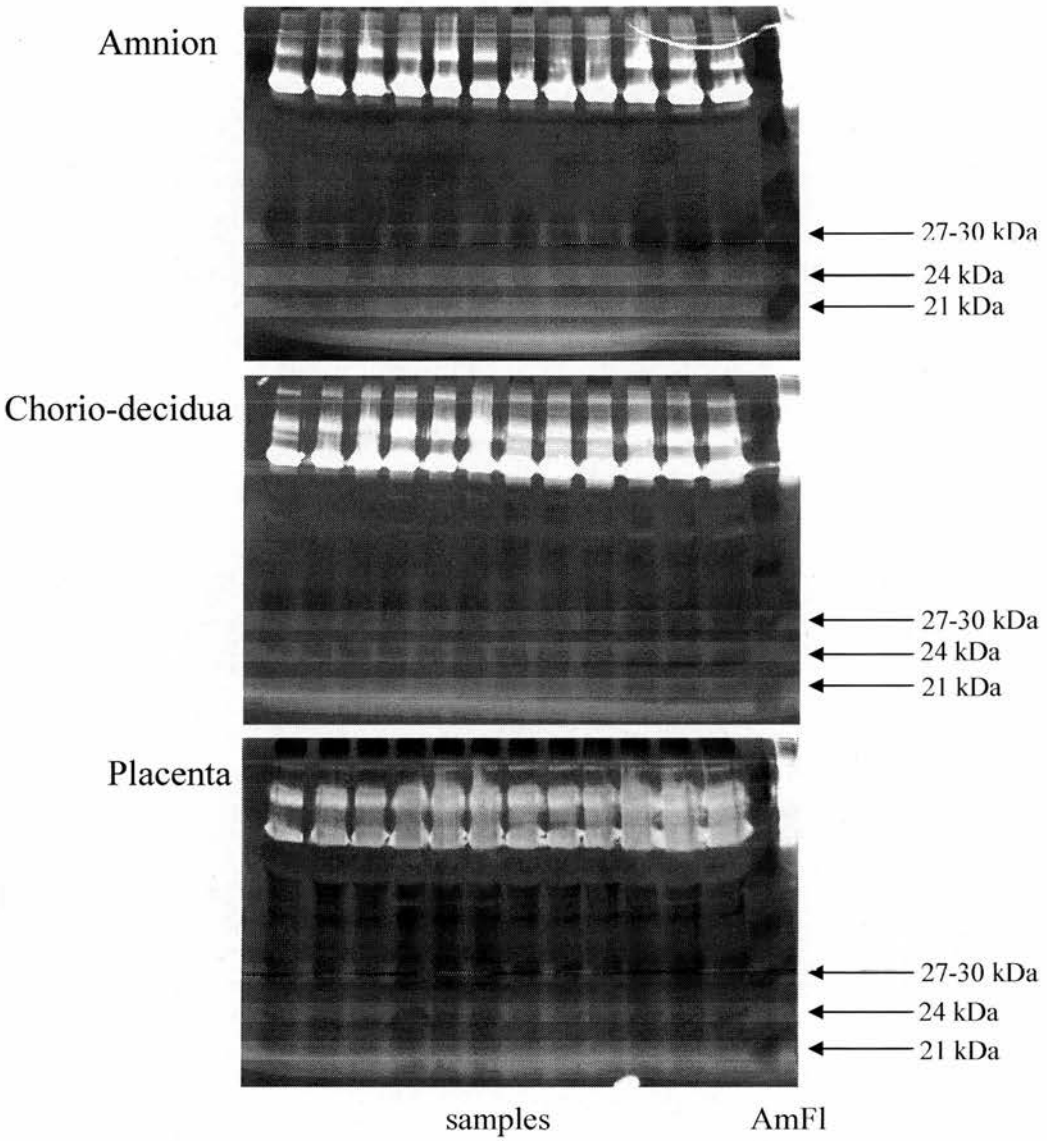


Figure 5.3: Sample reverse zymograms for amnion, chorio-decidua and placenta showing their production of TIMPs with activities of 27-30 kDa, 24 kDa and 21 kDa. The amniotic fluid (AmFl) loaded on the gels acts as a positive control as is has been characterized for TIMP activity.

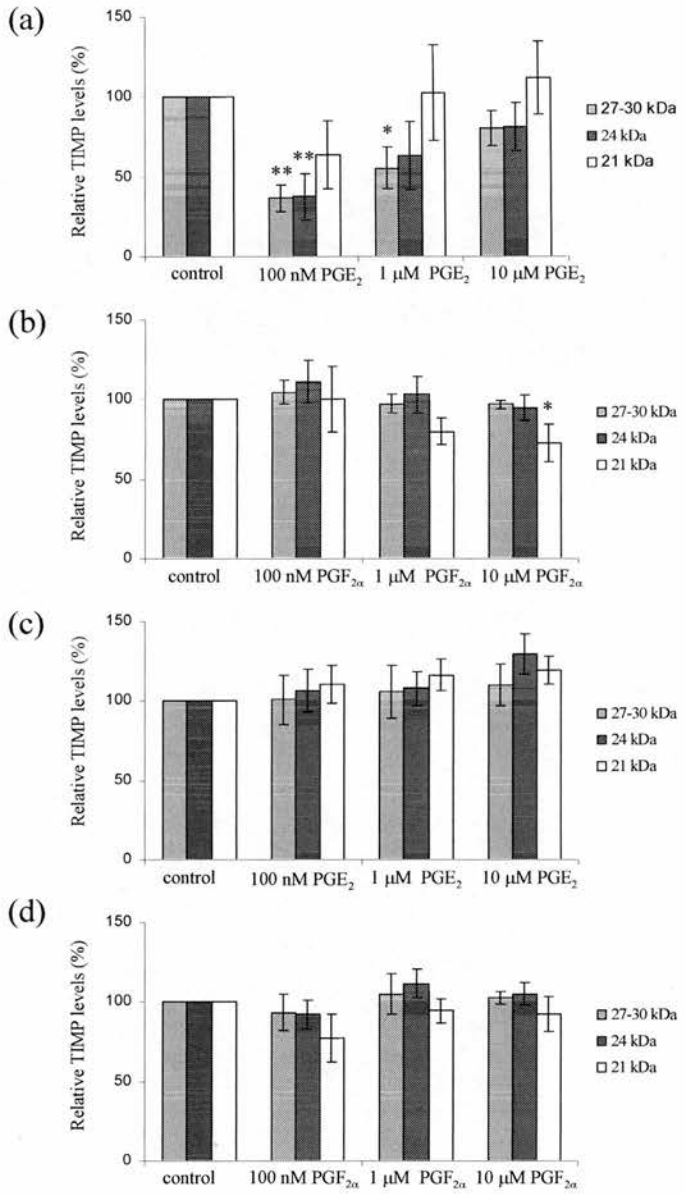


*Figure 5.4: The effects of incubation with 100 nM, 1 µM, or 10 µM PGE_2 (a and c) and $PGF_{2\alpha}$ (b and d) for 24 hr on TIMP production in amnion (a and b) and chorio-decidua (c and d) explants as determined by densitometric analysis of reverse zymograms. * and ** denote significance values of $p < 0.05$ and $p < 0.01$ respectively when compared to control untreated samples. n=5 patients with each treatment replicated 4 times.*

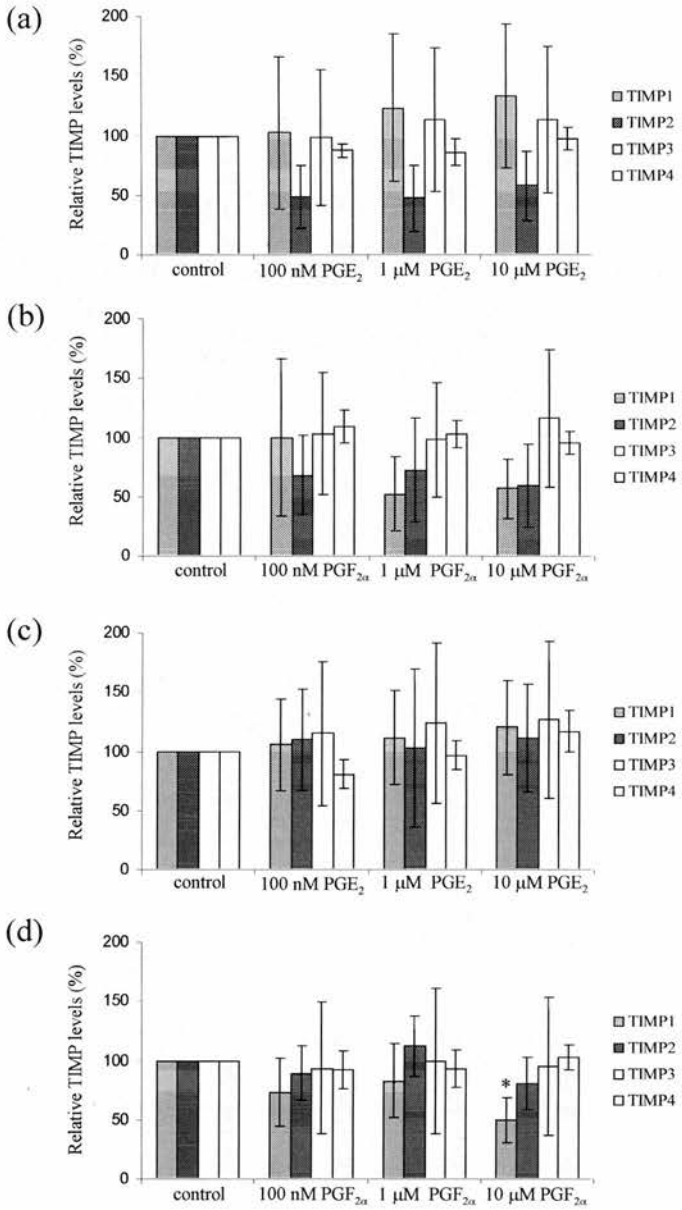


*Figure 5.5: The effects of incubation with 100 nM, 1 µM, or 10 µM PGE_2 (a and c) and $PGF_{2\alpha}$ (b and d) for 24 hr on TIMP production in amnion (a and b) and chorio-decidua (c and d) explants as determined by densitometric analysis of Western blots. * denote significance values of $p < 0.05$ when compared to control untreated samples. n=5 patients.*

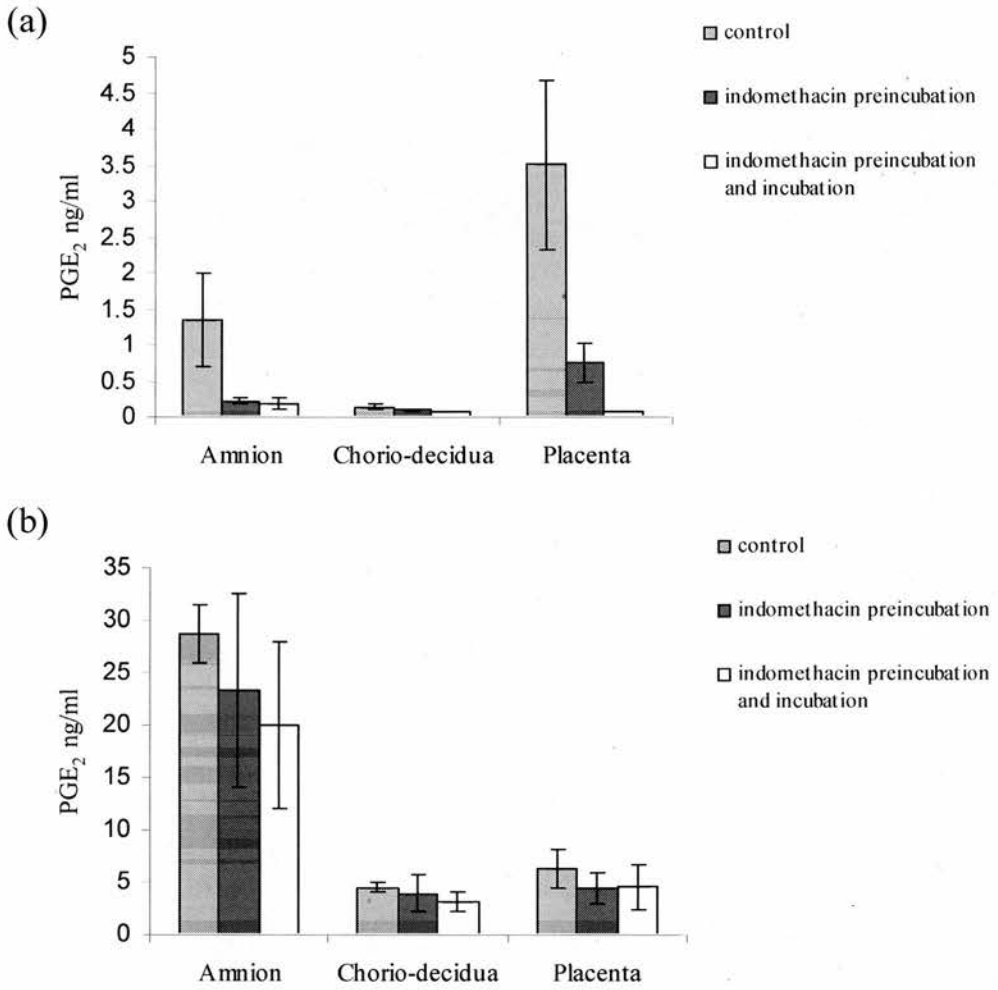


Figure 5.6: Effect of indomethacin on PGE_2 output in amnion, chorio-decidua and placenta explants in (a) samples with no PGE_2 treatment and (b) samples with 100 nM PGE_2 administered for 24 hours before assaying for PGE_2. n=3 patients.

In order to check that this indomethacin treatment had no adverse effects on the histology of the tissue, H and E staining was performed on fixed tissue blocks at different stages of the indomethacin treatment. The result of this immunohistochemical analysis is shown in *Figure 5.7*. No apparent differences were observed between tissue before and after exposure to a 2 hr indomethacin pre-treatment or a further 24 hr indomethacin treatment. As a consequence of this and the prostaglandin assay results, it was decided that a 2 hr indomethacin pre-treatment of tissue would be sufficient to inhibit endogenous prostaglandins to levels low enough that they would not mask the effects of PG treatment.

5.3.4 Effect of PGE_2 and $PGF_{2\alpha}$ on MMP-2 and MMP-9 with Indomethacin Pre-treatment

Results of zymogram analysis are shown in *Figure 5.8*. Graphs are presented with values as a percentage of the control at the relevant time. Levels of MMPs were higher after 24 hours compared to 8 hours, due to accumulation of the MMP in the media. In the amnion, PGE_2 treatment for 24 hr has caused MMP-2 levels to increase very slightly though significantly. In the chorio-decidua, $PGF_{2\alpha}$ has slightly but significantly increased MMP-9 after 8 hr and MMP-2 production after 24 hr treatment.

In JEG3 cells, it is mainly MMP-2 being produced (*Figure 5.9a*). A third band suspected to be another form of MMP-2 is visible below the latent and active forms of MMP-2 on the zymogram. This was confirmed to be a form of MMP-2 after it was detected by a specific MMP-2 primary antibody using Western blotting (*Figure 5.9b*). MMP-9 is being produced but at such low levels that detection is barely possible by zymography analysis, thus it is MMP-2 that has been analysed for the following experiments. After just 2 hr, $PGF_{2\alpha}$ significantly stimulated MMP-2 production to 135 % of control levels, and after 24 hr, PGE_2 raised MMP-2 levels to 125 % of the controls. A significant stimulation of MMP-2 to 180 % of controls was seen after treatment with IL-6 for 12 hr (*Figure 5.10*).

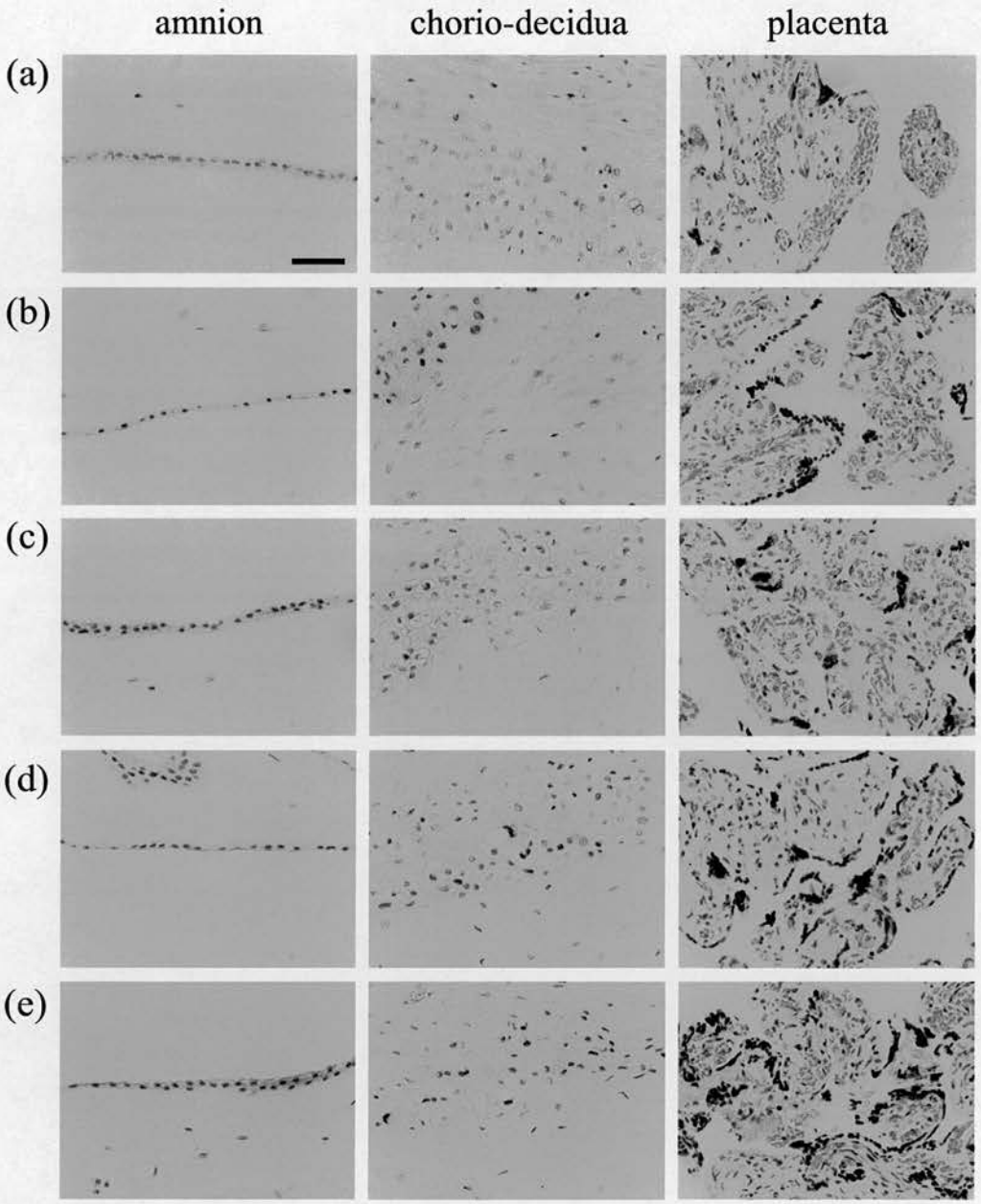


Figure 5.7: H&E staining to show effects of indomethacin pretreatment on histology of amnion, chorio-decidua and placenta samples. Treatments are (a) control samples after 2 hr steep with no indomethacin (b) after 2 hr incubation in indomethacin (c) after 2 hr with no indomethacin, then 24 hr without (d) after 2 hr with indomethacin then 24 hr without and (e) after 2 hr and 24 hr indomethacin treatment.

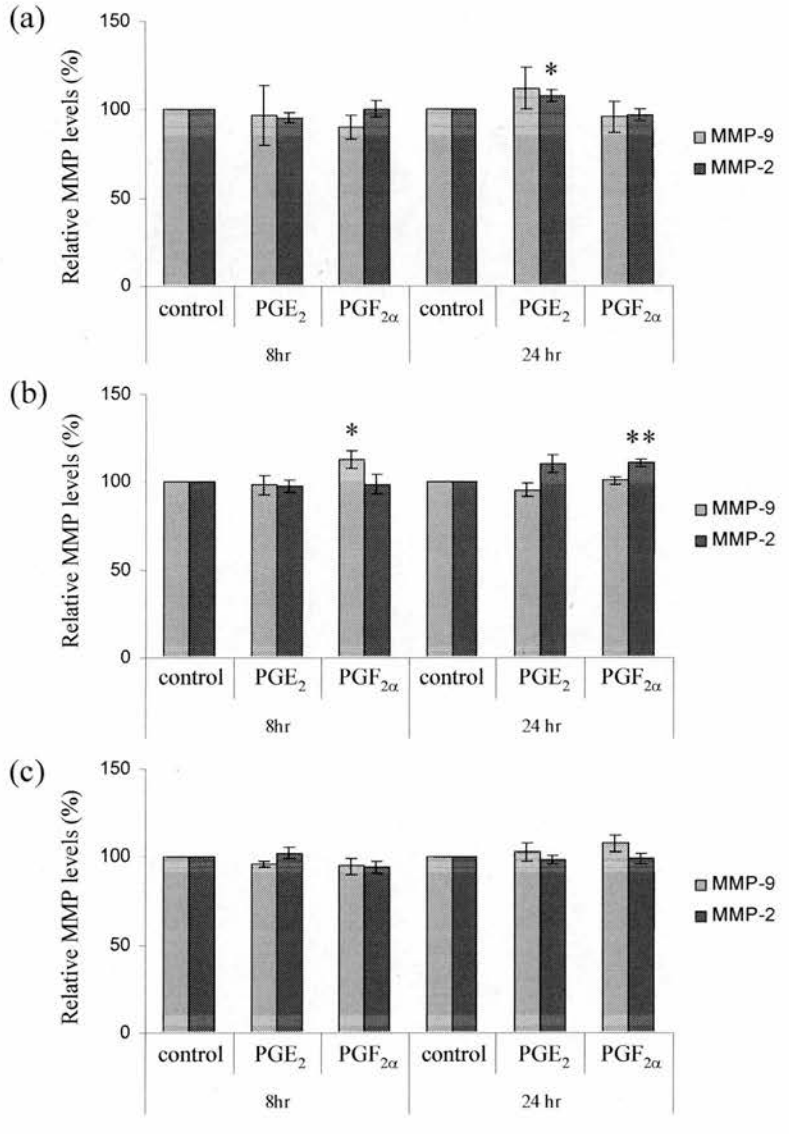


*Figure 5.8: MMP-2 and MMP-9 production by (a) amnion (b) chorio-decidua and (c) placenta after preincubation with indomethacin, then incubation with 100 nM PGE_2 or $PGF_{2\alpha}$ for 8 or 24 hr, as determined by densitometric analysis of zymograms. * and ** denote significance values of $p < 0.05$ and $p < 0.01$ respectively when compared to control untreated samples. n=4 patients, with each treatment carried out in triplicate.*

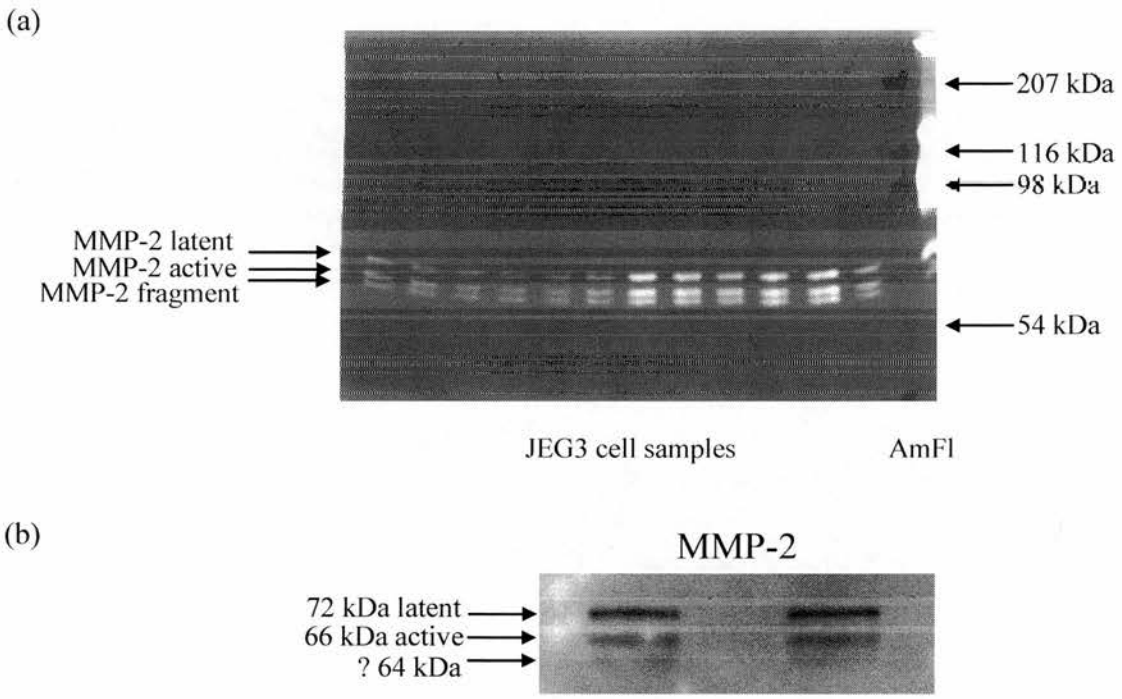


Figure 5.9: Representative zymogram (a) and Western blot (b) to show presence of latent and active forms of MMP-2 in cultured JEG3 cells.

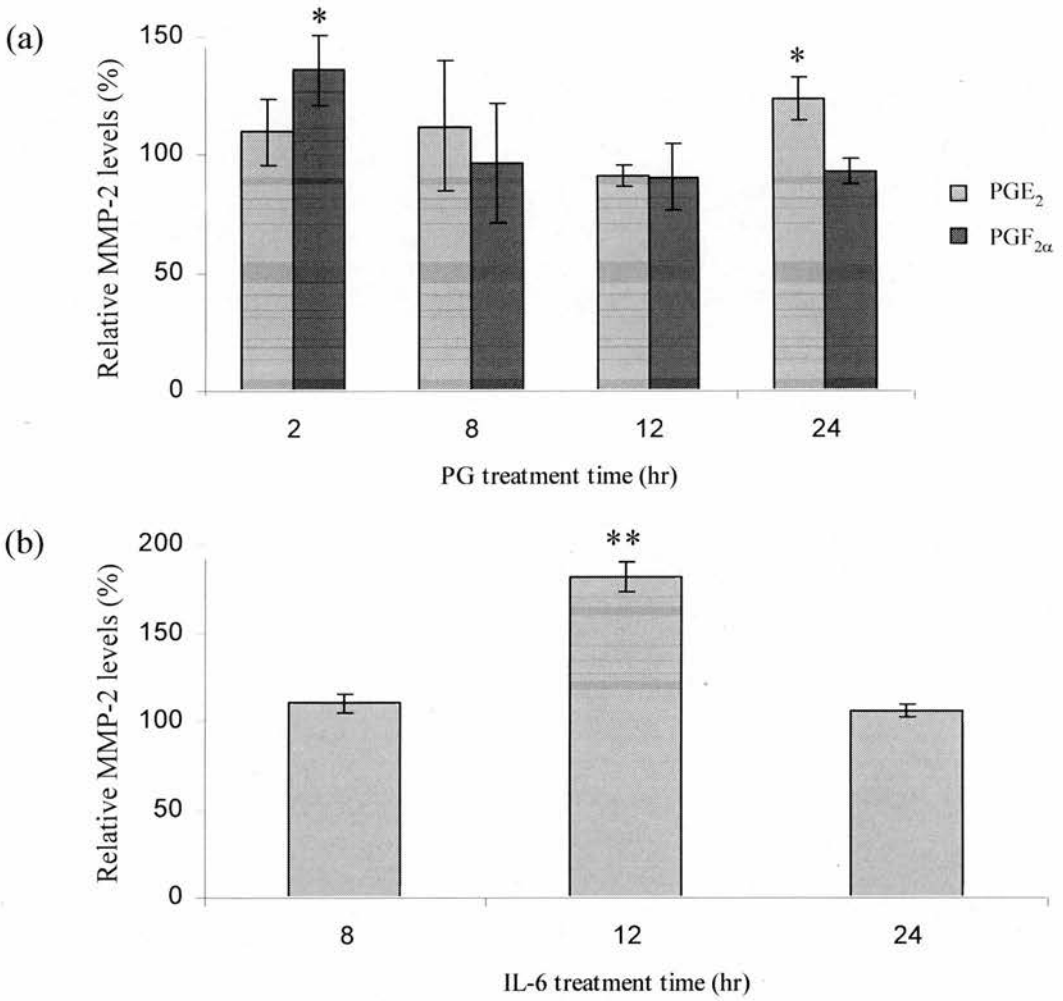


*Figure 5.10: MMP-2 production in JEG3 cells after preincubation with indomethacin, then a time course of incubations with (a) 100 nM PGE_2 or $PGF_{2\alpha}$ and (b) 0.5 nM IL-6 as determined by densitometric analysis of zymograms. * and ** denote significance values of $p < 0.05$ and $p < 0.01$ respectively when compared to control untreated samples. n=3 separate experiments with treatments carried out in triplicate.*

5.3.5 Effect of PGE_2 and $PGF_{2\alpha}$ on TIMP Production with Indomethacin Pre-treatment

The results of the analysis of TIMP production are shown for reverse zymography (*Figure 5.11*) and Western blotting (*Figure 5.12*). No significant alterations in TIMP levels are observed on addition of either prostaglandin. Due to antibody problems, levels of TIMP3 could not be measured.

5.3.6 Cellular MMP-2 and MMP-9 Content in JEG3 Cells

The latent form of MMP-2 was found in a zymogram of homogenised JEG3 cell samples (*Figure 5.13*). The same samples analysed showed trace levels of MMP-9 and also the active form of MMP-2 in addition to the latent form of MMP-2. All were expressed at lower levels than that seen in analysed media from these cells after 24 hr, suggesting most MMP-2 is secreted.

5.3.7 Effect of PGE_2 and $PGF_{2\alpha}$ on MMP-2 and MMP-9 mRNA

Results from lightcycler analysis of MMP-2 and MMP-9 mRNA in amnion, chorio-decidua and placenta stimulated with of PGE_2 and $PGF_{2\alpha}$ are shown (*Figures 5.14 and 5.15*). According to statistical analysis, none of the treatments produced any significant changes in MMP-2 or MMP-9 mRNA levels, however, a few changes are evident. In the amnion, after 8 hr treatment with $PGF_{2\alpha}$, MMP-2 and MMP-9 mRNA levels were elevated 160 % and 150 % respectively, compared to controls. In the chorio-decidua after 8 hr, of PGE_2 and $PGF_{2\alpha}$ had increased production of MMP-9 to 130 % that of controls. After 24 hr, these prostaglandins also elevated both MMP-2 and MMP-9 mRNA, for PGE_2 treatment to 170 % that of control and $PGF_{2\alpha}$ treatment to 150 % that of controls. In the placenta, MMP-2 mRNA production was almost doubled on addition of $PGF_{2\alpha}$ for 8 hr.

In JEG3 cells, MMP-2 mRNA production has been significantly stimulated by 4 hr treatments with IL-6, PGE_2 and $PGF_{2\alpha}$ by 180 %, 190 % and 210 % compared to that of

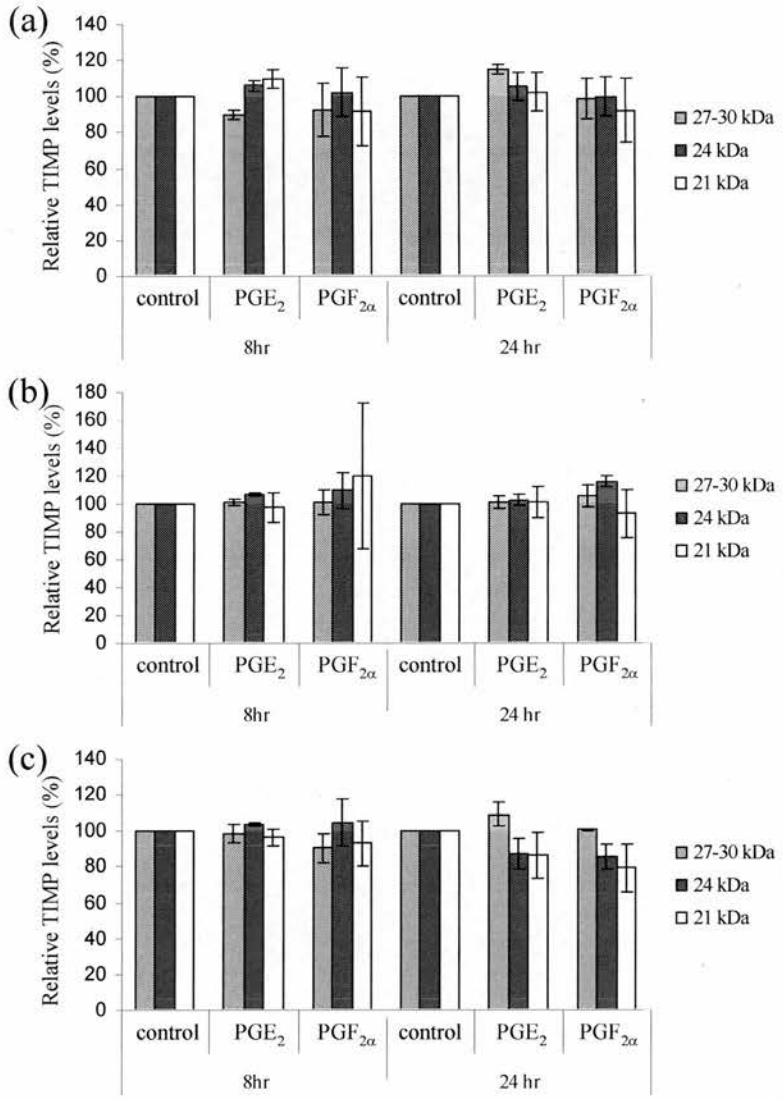


Figure 5.11: TIMP production by (a) amnion (b) chorio-decidua and (c) placenta after preincubation with indomethacin, then incubation with 100 nM PGE_2 or $PGF_{2\alpha}$ for 8 or 24 hr, as determined by densitometric analysis of reverse zymograms. n=4 patients with treatments carried out in triplicate.

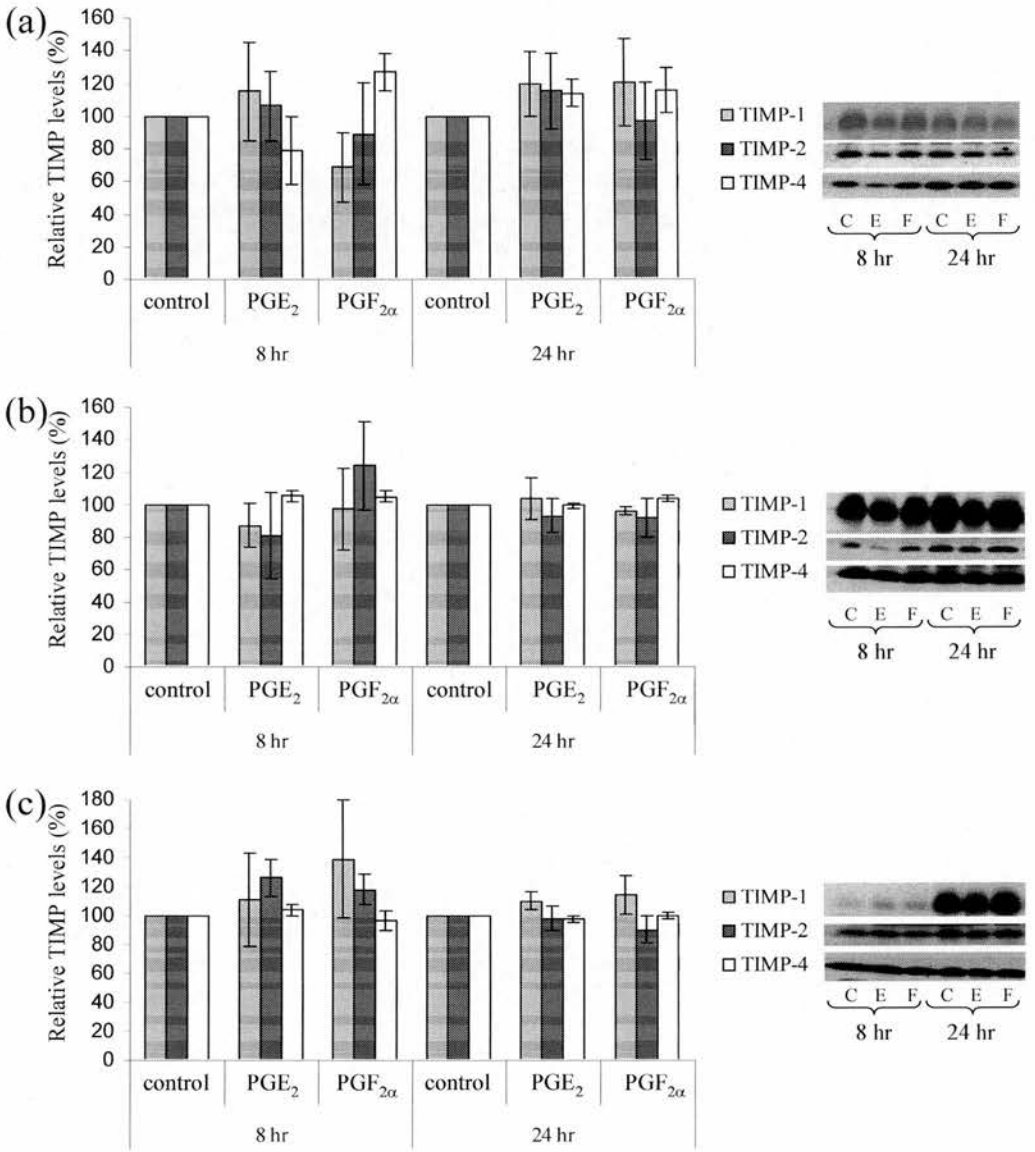


Figure 5.12: TIMP production by (a) amnion (b) chorio-decidua and (c) placenta after preincubation with indomethacin, incubation with 100 nM PGE_2 or $PGF_{2\alpha}$ for 24 hr, as determined by densitometric analysis of Western blots. Sample Western blots for TIMPs 1, 2 and 4 are shown alongside the graphs (C = control, E = PGE_2, F = $PGF_{2\alpha}$). n=4 patients.

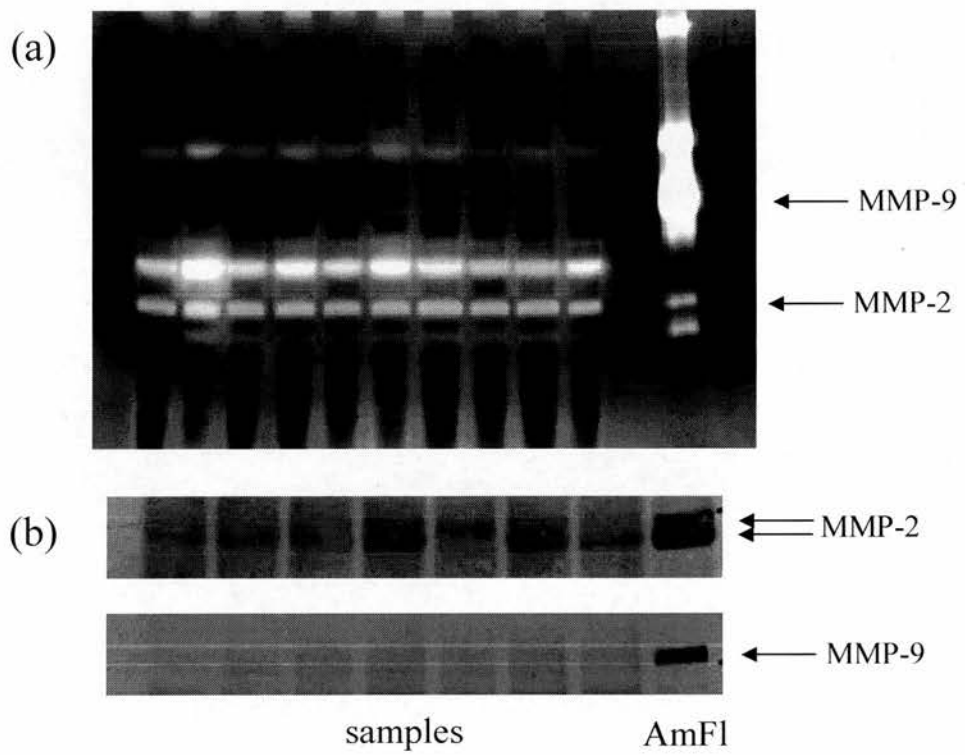


Figure 5.13: Representative zymogram (a) and Western blots (b) to show cellular content of MMP-2 and MMP-9 in JEG3 cells.

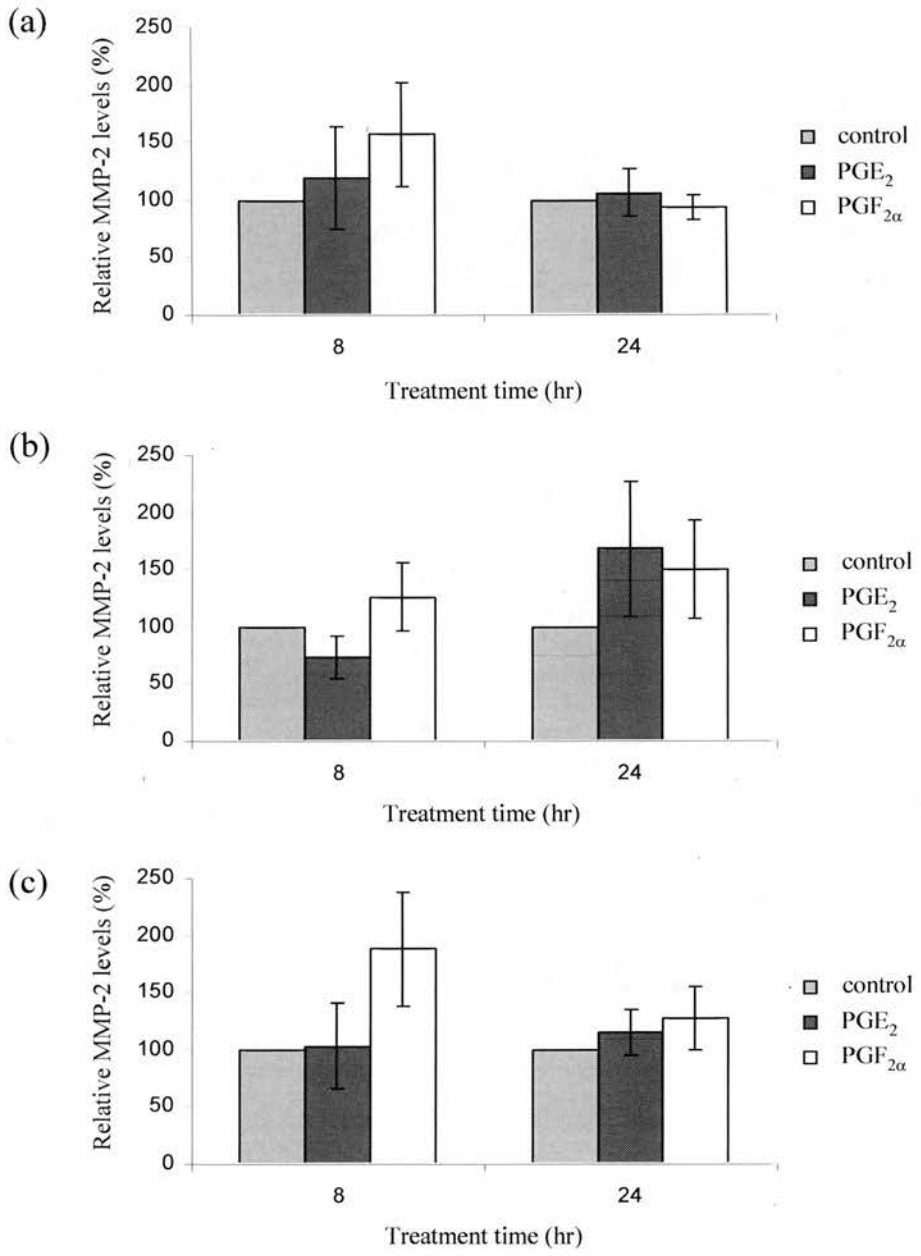


Figure 5.14: MMP-2 mRNA levels in (a) amnion (b) chorio-decidua and (c) placenta after preincubation with indomethacin, then a time course of incubations with 100 nM PGE_2 or $PGF_{2\alpha}$ as determined by quantitative PCR using the Lightcycler. n=3 patients with treatments carried out in triplicate.

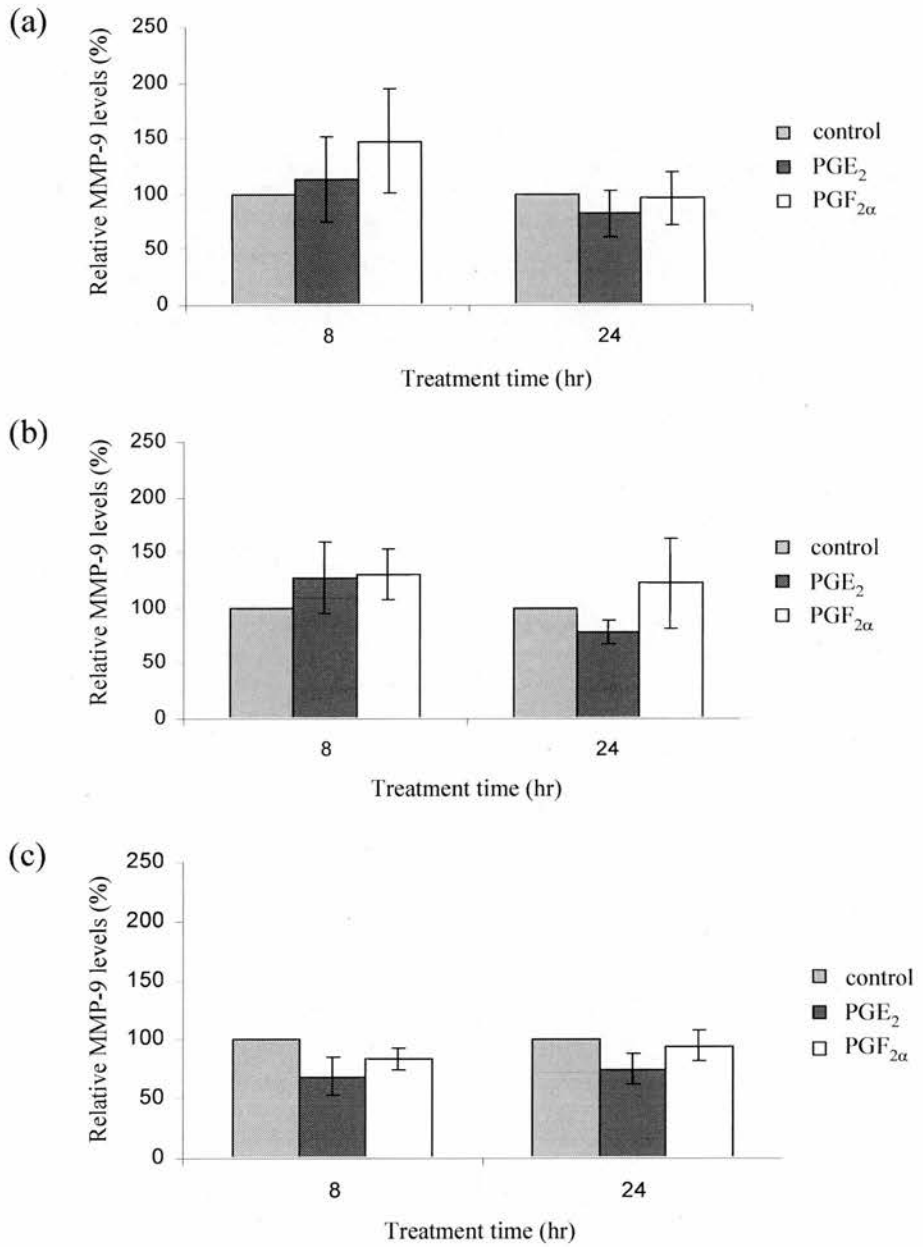


Figure 5.15: MMP-9 mRNA levels in (a) amnion (b) chorio-decidua and (c) placenta after preincubation with indomethacin, then a time course of incubations with 100 nM PGE_2 or $PGF_{2\alpha}$ as determined by quantitative PCR using the Lightcycler. n=3 patients with treatments carried out in triplicate.

controls, respectively (*Figure 5.16).* After 24 hours, PGE_2 and $PGF_{2\alpha}$ significantly increased MMP-2 mRNA levels by 175 % and 190 % that of controls.

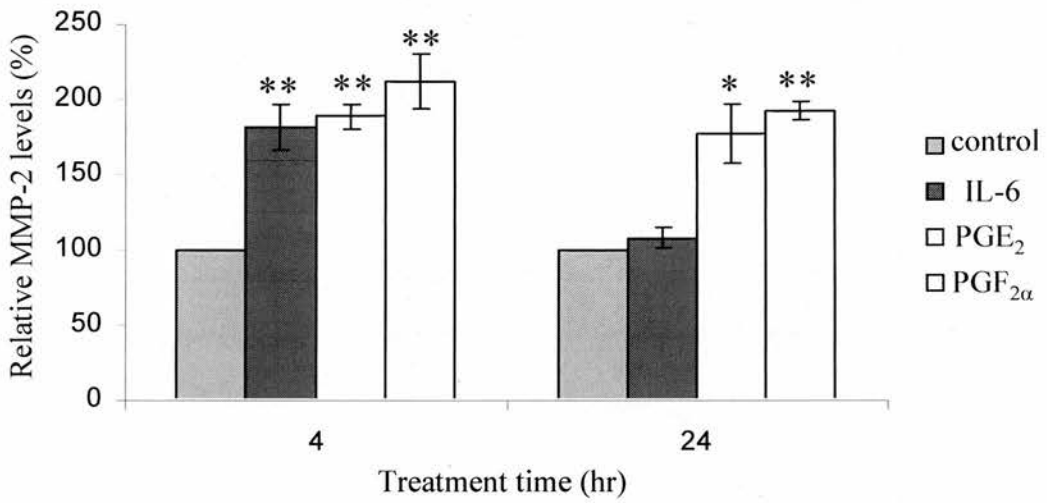


*Figure 5.16: MMP-2 mRNA levels in JEG3 cells after preincubation with indomethacin, then a time course of incubations with 100 nM PGE_2, 100 nM $PGF_{2\alpha}$ and 0.5 nM IL-6, as determined by quantitative PCR using the Lightcycler. * and ** denote significance values of $p < 0.05$ and $p < 0.01$ respectively when compared to control untreated samples. n=3 separate experiments with treatments triplicated.*

5.4 Conclusions

Degradation of fetal membranes and the adjacent decidua are essential processes for rupture of the fetal membranes at term and preterm. The gelatinases, MMP-2 and MMP-9 digest collagen in these tissues and are thus implicated in rupture of these membranes. It has been suggested that prostaglandins play a role in up-regulating the production of MMP-2 and MMP-9 in gestational tissues, and decreasing the production of the TIMPs, the endogenous inhibitors of MMPs (Ulug et al 2001). The results presented in this chapter show evidence that PGE_2 and $PGF_{2\alpha}$ can up-regulate the gelatinases and inhibit TIMP production in amnion, chorio-decidua and JEG3 cells. They have also demonstrated a prostaglandin stimulated up-regulation of MMP-2 mRNA production in JEG3 cells.

These experiments were designed using tissue from patients with term pregnancies who were not in labour and undergoing elective caesarean section. This was because it was assumed that this tissue would be "un-primed" by prostaglandins whose levels are known to increase in the fetal membranes and placenta during labour (Challis et al 2000, Thorburn 1991, Van Meir et al 1997). The addition of prostaglandins in theory then creates *in vivo* the conditions which prostaglandins contribute to during labour. It would have been interesting to compare spontaneous labour tissue, and also preterm patients, but due to ethical and practical considerations, these experiments could not be performed. To investigate the effect of prostaglandins on MMP and TIMP production, it was decided that a preincubation of indomethacin would be necessary to obliterate the effects of endogenous prostaglandins which may mask the effects of the treatments. It was unclear whether the results from experiments with no indomethacin pre-treatment would be a true representation of the effect of prostaglandin stimulation on MMPs. Indomethacin is a commonly used non steroidal anti-inflammatory drug (NSAID). It is a non-selective COX-inhibitor, so it reduces prostaglandin synthesis. It appears from these results to not have any damaging histological effects on the tissue, and to reduce endogenous PGE_2 levels to much lower than the doses used, so it was considered an

effective step in the experiment. In similar studies, indomethacin has been found to reduce the secretion of latent MMP-2 and MMP-9 in fetal membranes but not decidua, and increase the production of TIMP- in fetal membranes but not decidua, suggesting the reduction in prostaglandins levels is reducing the prostaglandin stimulation of MMP production (McLaren et al 2000b, Ulug et al 2001).

MMP-2 and MMP-9 have been shown to be released by human fetal membranes and placental tissue. Analysis of JEG3 cells show that these produce predominantly MMP-2, with small amounts of MMP-9 and that most MMP is secreted and not stored within the cell. Any large changes in MMP and TIMP production upon prostaglandin treatment have not been obvious from the experiments in this chapter. It appears that, with the indomethacin pre-treatment, the only significant effect is a very small stimulation of MMP-2 by PGE_2 after 24 hours, $PGF_{2\alpha}$ stimulating MMP-9 production after 8 hours, and MMP-2 production after 24 hours. In these same samples, TIMP levels are unaffected by the addition of PGE_2 and $PGF_{2\alpha}$. The results of the JEG3 experiments showed small but significant changes in MMP-2 production after 2 hours with $PGF_{2\alpha}$ and 24 hours with PGE_2. MMP-9 production within these cells was minimal so any measurement using quantification of zymography would have been inaccurate. TIMP measurements by reverse zymography and Western blotting with indomethacin pretreatment showed no changes with prostaglandin treatment. The reverse zymography experiments with no indomethacin pretreatment suggest that PGE_2 is inhibiting TIMP activities at 27-30 kDa and 24 kDa in the amnion. However, these results were not consistent with Western blotting of the same samples, so the true nature of PGE_2 and $PGF_{2\alpha}$ regulation of TIMPs in fetal membranes remains unclear. TIMPs in the JEG3 cells were expressed at such low levels that they were barely detectable by Western blotting or reverse zymography, so quantification was not possible. Analysis of TIMP mRNA by PCR could have given a useful insight into this study.

These results go alongside results from recent experiments, in which PGE_2 was found to stimulate MMP-9 secretion in cultured fetal membranes in a dose dependent manner,

with significant increases being at 1, 10 and 100 nM PGE_2, where over 2-fold increases in MMP-9 secretion are observed (McLaren et al 2000b). $PGF_{2\alpha}$ had a similar effect in cultured decidual samples, causing around a 2-fold increase in MMP-2 and MMP-9 production, a large significant increase in the active form of MMP-2, and a 70 % reduction in TIMP-1 levels (Ulug et al 2001). These authors found that $PGF_{2\alpha}$ had no effect on the gelatinases and TIMP-1 in the amnion and chorio-decidua.

It is possible that the culture model used in this chapter may have not been effective in allowing MMP protein stimulation with the prostaglandins. There may have been some limiting factor or inhibition mechanism within the culture set-up, such as the composition of nutrients in the media, which did not allow for the intracellular pathways required to occur, or for translation to take place. So, as well as protein levels, MMP-2 and MMP-9 mRNA levels were monitored upon treatment with prostaglandins. In the amnion, chorio-decidua and placenta, the prostaglandins had no effect on MMP-9 mRNA production. However, MMP-2 mRNA levels were increased slightly by $PGF_{2\alpha}$ after 8 hr in amnion and placenta culture, and by PGE_2 and $PGF_{2\alpha}$ after 24 hr in chorio-decidua, though these results were not statistically significant. Both PGE_2 and $PGF_{2\alpha}$ significantly stimulated MMP-2 mRNA production after 4 and 24 hr treatments in JEG3 cells.

IL-6 effects on MMP-2 and MMP-9 production were investigated in JEG3 cells. Here, latent MMP-2 production was stimulated nearly 2-fold after 12 hours of incubation with 0.5 nM IL-6. IL-6 also stimulated MMP-2 mRNA levels by nearly 2-fold after only 4 hours. Using the JEG3 cells as a model of chorion trophoblast cells, this suggests that IL-6, and possibly other proinflammatory cytokines may be involved in the rupture of fetal membranes and placental detachment by stimulating the degradative MMP enzymes.

The distribution of MMP-2 and MMP-9 within uterine and gestational tissues is quite distinct. MMP-2 is localised to the amnion mesenchyme, the chorion trophoblast, the

decidua parietalis and blood vessels in the placental villi. MMP-9 is detected in amnion epithelium, chorion trophoblast, decidua parietalis and placental syncitiotrophoblasts (Xu et al 2002). This variation may be due to the different collagen components of these cell layers. Both MMP-2 and MMP-9 degrade collagen IV, but only MMP-2 digests collagen I, which is found in the amnion mesenchyme layer (Bryant-Greenwood 1998). These differential localisations of MMP-2 and MMP-9 may be important for the rupture of the fetal membranes and placental separation.

MMP-2 and MMP-9 production and activation are also regionally controlled within the fetal membranes. A zone of altered morphology (ZAM) has been identified as a restricted area of the fetal membranes after their spontaneous rupture (Malak & Bell 1994b). This area is structurally weaker due to degradation of ECM. Similar alterations have been identified in the fetal membranes in the lower uterine segment overlying the cervix (McLaren et al 1999). Here, pro-MMP-9 levels are higher prior to labour compared to fetal membrane tissue examined from the midzone and the placental edge, whilst pro-MMP-2 levels are not different. With the onset of labour, the pro and active forms of both MMP-2 and MMP-9 increase in all regions (McLaren et al 2000a). The authors propose that before labour, the increase in pro-MMP-9 may provide a store of potential activity that, if activated, possibly by the actions of labour it self, will preferentially weaken the membrane to increase its susceptibility to labour. Prior to delivery it is weakened by the generalised increased activity of MMPs and represents the initial site of rupture that is identified as the ZAM. Changes in control of this mechanism of MMP-9 action could result in preterm premature rupture of the membranes.

Little is known about the contribution of TIMPs to tissue remodelling during labour. The technique of zymography by which most research groups detect active and latent forms of MMP, only detects MMPs which are unbound. Thus, the true enzymatic activity may not be measured using this technique, if the active form of the enzyme is bound and inhibited by a TIMP. A precise balance of TIMPs and MMPs is necessary to control actual enzymatic activity. TIMPs 1-4 have all been detected in amniotic fluid and

amnion, chorion, decidual and placental tissue (Fortunato et al 1998, Riley et al 1999b), although TIMP-4 was expressed at very low levels in amniotic fluid. Levels of TIMPs 1-3 were significantly higher in elective caesarean section amniotic fluid samples than those from spontaneous labour, showing a decrease in levels with the onset of labour (Riley et al 1999b). There were relatively low levels of TIMPs in the amnion compared with the other tissues mentioned, suggesting the amnion is not the source of TIMPs in the amniotic fluid.

A change in the precise balance of MMP and TIMPs has been implicated in preterm premature rupture of the fetal membranes (PPROM). Elevated amniotic fluid concentrations of MMP-9 were found to be higher in PPROM compared to preterm labour (Romero et al 2002) and PROM compared to term labour (Vadillo-Ortega et al 1996). MMP-2 and MMP-9 latent and TIMP-free active forms were both elevated in amniotic fluid in PPROM compared to preterm and term labour (Fortunato et al 1999). Furthermore, TIMP-1 levels were found to be lower in amniotic fluid from term PROM than term labour (Vadillo-Ortega et al 1996). In amniochorion membranes, lipopolysaccharide can elevate the expression and release of both gelatinases, and decrease TIMP-2 levels, shifting the ratio in favour of gelatinase activity (Fortunato et al 2000). Shifting this balance increases membrane degradation by the MMPs, predisposing the individual to PPROM.

The incidence of preterm birth and PPROM differs among ethnic groups with the risk of preterm birth for African Americans being more than twice that of Caucasians (Hoffman & Ward 1999). PPROM is also more likely to precede spontaneous labour in African Americans (Ventura et al 2001). This suggests genetic factors are involved in PPROM. MMP-9 expression is primarily regulated at the transcription level. Several polymorphisms have been identified in the MMP-9 gene, and these are postulated to play a role in PPROM due to the effects of MMP-9 on fetal membranes (Ferrand et al 2002). This group have investigated two polymorphisms in the proximal promoter: a variable CA repeat, and a single nucleotide polymorphism (SNP). In African American

women, the presence of a 14 CA-repeat displayed significantly increased activity in isolated amnion epithelial cells compared to a 20 CA-repeat. The 14 CA-repeat is more common than any other variation and is more represented in neonates from pregnancies complicated by PPROM than others. Despite this, this allele is less common in African Americans than Caucasians (St Jean et al 1995), so cannot be used to explain the higher incidence of PPROM in these women.

Other MMPs may also be involved be involved in parturition, and may also be affected by prostaglandins and cytokines. MMPs -1, -7 and -8 have all been implicated in PPROM (Maymon et al 2000a, Maymon et al 2000b, Maymon et al 2000c). MMP-1 is present in amniotic fluid and increases in this were associated with PPROM, in the presence and absence of intrauterine infection, though no changes in MMP-1 were associated with term or preterm labour. MMP-7 concentrations in the amniotic fluid increase with advancing gestational age. Preterm gestations showed an increase in MMP-7 with microbial invasion of the amniotic cavity. MMP-8 concentrations in amniotic fluid increased with spontaneous labour at term and preterm. Spontaneous rupture of the membranes was also associated with elevations of MMP-8 in preterm but not term gestations. MMP-13 has been shown to be present in amniotic fluid and constitutively expressed in fetal membranes, and not to be associated with PPROM (Fortunato et al 2003).

An extracellular matrix metalloproteinase inducer (EMMPRIN) has been discovered and characterised and is shown to be a glycoprotein and a member of the immunoglobulin superfamily (Biswas et al 1995). As well as playing a role stimulating MMP production in cancer systems, EMMPRIN has recently been implicated in parturition (Li et al 2004). EMMPRIN was detected in term placenta and fetal membranes in three forms: 40 and 65 kDa (glycolsylated protein) and 30 kDa (nonglycosylated). While there were no changes in total EMMPRIN, the relative amount of the 65 kDa form significantly increased in term labour amnion and chorio-decidua compared with non-labour. This shows that MMP production can be stimulated in the fetal membranes and placenta,

which could lead to facilitation of fetal membrane rupture and detachment of the placenta and fetal membranes from the maternal uterus at the time of parturition. Also, glycosylation could be important for functional activity of EMMPRIN at parturition.

In conclusion, MMP-2 and MMP-9 have been suggested to be involved in rupture of the fetal membranes, and PGE_2, $PGF_{2\alpha}$ and IL-6 have been implicated in the up-regulation of these enzymes at the time of this rupture. Indomethacin has been shown to diminish the prostaglandin levels in gestational tissues. These mechanisms are likely to be important in PROM at term and preterm, and indomethacin could be a useful agent to delay premature rupture of the fetal membranes.

Chapter 6: General Discussion

6.1 Synopsis of Findings Presented in this Thesis

These studies have highlighted some of the pathways by which PGE_2 and $PGF_{2\alpha}$ can regulate production and activation of MMP-2 and MMP-9, which are implicated in the rupture of the fetal membranes at term and preterm. These findings are described in a schematic diagram in *Figure 6.1*, with respect to JEG3 cells.

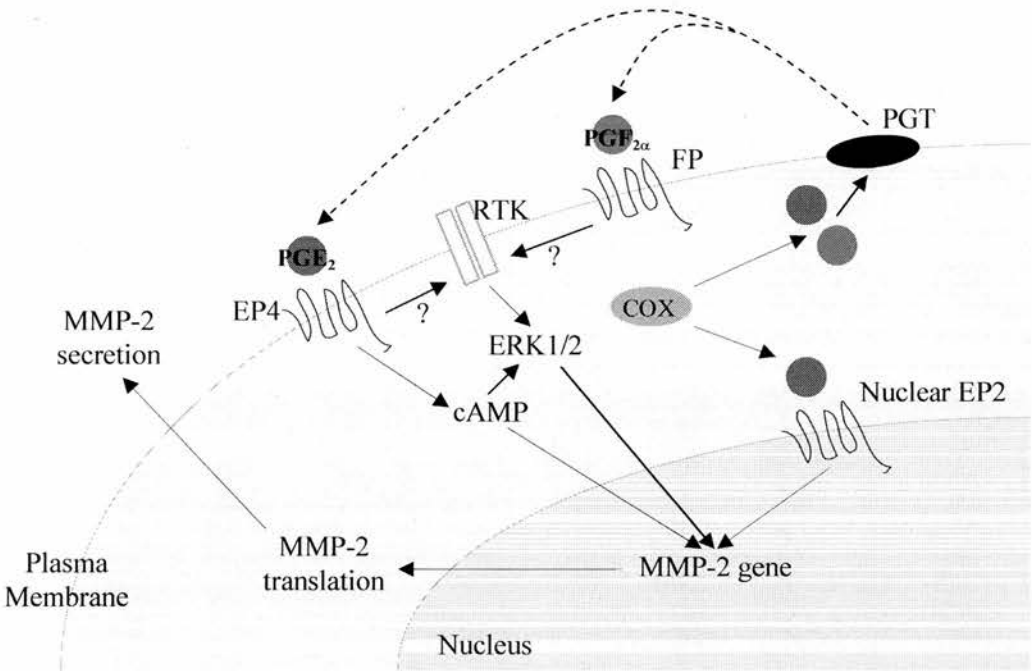


Figure 6.1: Regulation of autocrine and paracrine PGE_2 and $PGF_{2\alpha}$ signalling via EP and FP receptors in JEG3 cells, and their effects on MMP-2. RTK; recetor tyrosine kinase, PGT; prostaglandin transporter, COX; cyclo-oxygenase, ERK; extracellular signal regulated kinse, MMP; matrix metalloproteinase.

The mRNA for the prostaglandin receptor EP4 was detected in amnion, chorio-decidua and placenta, and FP mRNA found in chorio-decidua and placenta samples. Interestingly, mRNA for EP2 was undetected in fetal membranes and placenta. EP2, EP4, FP, and also PGDH, were immunolocalised to the amnion epithelium and fibroblast layers, the reticular and trophoblast layers of the chorion, decidual glandular epithelial cells, stromal cells and vascular endothelial cells, syncytiotrophoblasts of the placenta, myometrial smooth muscle cells, and JEG3 cells. Immunostaining of EP2, EP4 and FP was seen predominantly in the plasma membrane, with nuclear staining of myometrial cells. EP2 was also visualised on JEG3 cell nuclear membranes.

Signalling pathways occurring via EP2, EP4 and FP in human fetal membranes and JEG3 cells were also investigated. It was shown that PGE_2 stimulates the cAMP/PKA pathway via the EP2 receptor in amnion tissue, and via the EP4 receptor in JEG3 cells, with no effect on chorio-decidua samples. The ERK pathway was found to be activated by PGE_2 and $PGF_{2\alpha}$ in JEG3 cells, but not in amnion or chorio-decidua samples. The PGE_2 effect was mediated via the EP4 receptor, and the $PGF_{2\alpha}$ effect occurred via the FP receptor. An inhibitor of MEK inhibited the activation of the pathway, though inhibitors of PLCβ and an EGFR kinase did not, showing that this pathway is not activated via PLCβ or by transactivation of the EGFR. Furthermore, the IP_3 pathway was not stimulated by $PGF_{2\alpha}$ in JEG3 cells.

The effect of prostaglandins, PGE_2 and $PGF_{2\alpha}$ on MMP-2 and MMP-9 mRNA, and the latent and active forms of the protein, and also TIMP levels and activity, was studied in term fetal membranes and placenta, and in JEG3 cells. In the fetal membranes and placenta, there were no clear prostaglandin related changes in MMP-2 and MMP-9 production or activation, or in TIMP production. There were also no reported changes in MMP-2 or MMP-9 mRNA levels with this treatment. However, in the JEG3 cells, the latent form of MMP-2 was significantly up-regulated by $PGF_{2\alpha}$ after 2 hr, and by PGE_2

after 24 hr. PGE_2 and $PGF_{2\alpha}$ both also increased MMP-2 mRNA production after 8 and 24 hr.

6.2 Clinical Relevance of Findings Presented in this Thesis

Premature rupture of the fetal membranes (PROM) is defined as rupture of the membranes before the onset of labour. Preterm premature rupture of the membranes (PPROM) is defined as rupture of the membranes before 37 weeks gestation. PPROM occurs in around 5-10 % of all pregnancies, and accounts for 30-40 % of all preterm deliveries (Parry & Strauss 1998). It is associated with increased risk of infection, when the time between rupture and delivery is delayed. Infection has also been implicated as a cause of PROM and PPROM.

Rupture of the membranes was once attributed to physical stress, however there is evidence to date suggesting that membrane rupture may also be due to biochemical processes, including degradation of the collagen within the ECM of the fetal membranes. The MMP family of proteinases are capable of degrading virtually every component of ECM, and have thus been associated with rupture of the membranes (Fortunato et al 1999, Vadillo-Ortega et al 1995, Vadillo-Ortega et al 1990). The collagens in the fetal membranes are of both the interstitial types, I, III and V, and type IV collagen which is the major component of the basement membrane (Aplin et al 1985, Bryant-Greenwood & Yamamoto 1995). This basement membrane is associated with the amnion epithelium and chorion trophoblast layers particularly, and also the reticular layer of the chorion. These collagens are particular substrates for the gelatinases, MMP-2 and MMP-9 (Lei et al 1999). Levels of MMP-2 and MMP-9 in the fetal membranes and amniotic fluid have been shown to increase during the process of labour and PROM, and levels of their endogenous inhibitors, the TIMPs, to decrease (Arechavaleta-Velasco et al 2002, Fortunato et al 1999, Fortunato et al 2000, Goldman et al 2003, Locksmith et al 1999, Maymon et al 2000c, McLaren et al 2000a, Riley et al 1999b, Romero et al 2002, Uchide et al 2000, Vadillo-Ortega et al 1995, Vadillo-Ortega et al 1996, Vadillo-

Ortega et al 2002, Xu et al 2002). Prostaglandins, particularly PGE_2 and $PGF_{2\alpha}$ have been implicated in labour, and reduced potential for prostaglandin degradation has been seen in the fetal membranes overlying the lower uterine segment, prior to labour (Van Meir et al 1997). Prostaglandins have been shown to stimulate the production of MMPs in fetal membranes and decidua (McLaren et al 2000b, Ulug et al 2001).

The results of the immunolocalisation studies in this thesis indicate potential sites of action of the prostaglandins. These sites correlate to the sites of prostaglandin synthesis, as shown by studies of COX expression and PG release, and metabolism, as shown from the PGDH immunolocalisation performed here, and also from an extensive series of studies by others (see section 3.4). EP2, EP4 and FP were all present in the fetal membranes prior to and during labour at term. The only apparent differences in distribution of any of these prostaglandin receptors, was the absence of FP in the amnion epithelium of preterm samples, and the absence of EP4 in preterm labouring samples. The appearance of FP in the amnion epithelium at term could represent a site of action of $PGF_{2\alpha}$ at term, though it most likely would not influence PROM. The absence of EP4 in amnion epithelium in preterm labouring samples is not clear, due its presence in term labouring and non-labouring samples. These studies only clarify the distribution and localisation of the receptors, and do not actually quantify receptor levels, so it is not clear whether levels are up-regulated at labour or term. Increased concentrations of prostaglandins available to act on their receptors could be the key to increased prostaglandin action at labour, or PROM. The presence of the EP2, EP4 and FP in the placenta and myometrium suggest that these are sites for PG action also. It is well known that PGE_2 and $PGF_{2\alpha}$ are involved in the processes of cervical ripening and dilatation and myometrial contractility, respectively (Calder 1994, Carbillon et al 2001, Winkler 2003), and prostaglandins have also been implicated in placental separation (Gross et al 1987) so the localisation of the prostaglandin receptors here is expected. The presence of the receptors on the nuclear membrane in myometrial smooth muscle cells suggests that intracrine signalling may play a role in prostaglandin action on these cells.

However, further experiments would have to be carried out to test the functionality of these receptors and the intracellular signalling pathways mediated via them.

Examining the signalling pathways elucidated via EP2, EP4 and FP in gestational tissues indicated whether the receptors were functionally active in these tissues. The results showed that EP2 was functionally active in amnion, and both EP4 and FP were in JEG3 cells. Stimulation of cAMP production in the amnion and JEG3 cells shows a prostaglandin mediated activation of the cAMP/PKA pathway, which can affect transcriptional activity of target genes within the nucleus. The ERK pathway was also activated by PGE_2 and $PGF_{2\alpha}$ in JEG3 cells. This can also influence gene transcription and activation of proteins by changes in phosphorylation state. This pathway would have been expected to be activated in the human fetal membranes as well. However, no changes were identified in ERK phosphorylation after treatment with PGE_2 and $PGF_{2\alpha}$, indicating that prostaglandins do not activate the ERK pathway in this tissue.

The downstream effects of these intracellular signalling pathways have not been clarified in the fetal membranes. cAMP/PKA and ERK can act on several gene regulatory proteins and transcription factors to target transcription and activation of many proteins. However, it is likely that MMPs are one of these targets. Previous studies have shown that PGE_2 induced activation of the cAMP pathway can up-regulate MMP-2 and MMP-9 production (Lyons et al 2002). ERK1/2 phosphorylation has been shown to up-regulate MMP-2 production in JEG3 cells (Andrassy et al 2004), and the studies in this thesis have shown that PGE_2 and $PGF_{2\alpha}$ stimulate ERK1/2 phosphorylation and MMP-2 production in these cells. Putting these together, it follows that PGE_2 and $PGF_{2\alpha}$ exert their stimulatory effect on MMP-2 via EP4 and FP and phosphorylation of the ERK pathway *(Figure 6.1)*. This pathway is likely to be involved in PROM. Use of receptor antagonists to the EP4 and FP receptor, which inhibit the PG induced activation of the cAMP/PKA and ERK pathways could be of potential use in blocking the PG induced activation of MMP-2 as a treatment to prevent PROM.

6.3 Models used in this Thesis for Investigating Parturition

As discussed in section 1.2.4, there are no ideal animal models for the studies of parturition. Several methods of non-invasively investigating parturition in humans have been derived. In this thesis, tissue explants of fetal membranes and placenta have been collected and cultured, alongside work carried out in a cell-line. The benefits and advantages of these models are discussed in this section.

6.3.1 Tissue Explant Model

The *in vitro* tissue explant model used in the thesis has been described before (Brennand et al 1995) and similar methods have also been used (Fortunato et al 1994, Mitchell & Powell 1984). Mounting the primary tissue explants on capillary matting allows the tissue to remain oriented as it would *in vivo*, retaining the arrangement of all cell types within the tissue. This maintains all the autocrine and paracrine relationships within the tissue, without it rolling up, creating new cell-cell interactions within the culture. It also allows tissue to remain histologically similar for up to 72 hours. It is also simple, allowing for easy aspiration and replacement of culture medium. Isolating primary cells, such as chorion trophoblast cells, and culturing them is another means by which investigations could have been carried out.

6.3.2 JEG3 Cells as a Model of Chorion Trophoblast

The JEG3 choriocarcinoma cells are derived from human trophoblast cells. The cells stain positive for cytokeratin, indicating that they are of epithelial origin, as expected. They can be used as a model of chorion trophoblast cells with respect to parturition (Marvin et al 2000, Moise et al 1986), the use for which has been described in this thesis. To ascertain if they function in a similar manner to trophoblast cells from human chorion at parturition, these cells would have to be isolated and experiments carried out to compare the intracellular pathways and functional responses. In this thesis, experiments show that the actions in these cells are not the same as that of chorio-decidua explants. This is likely due, at least in part, to the variety of cell types in the

human chorion layer, and in these explants with a small amount of adherent decidua. As discussed in chapter 4, opposing intracellular signalling pathways may be being activated via different receptors in these different cells. The PGE_2 stimulation of cAMP production seen in JEG3 cells was not observed in chorion explants and that could be due to co-expression of EP receptors with opposing effects. These cells are derived from a choriocarcinoma, so they are different from chorion trophoblast cells is some aspects of cell function, for example the control of the cell cycle pathway. They have also been stored and maintained in culture through multiple passages, which may also alter their functionality. The explant is more representative of the tissue as a whole due to all cell types and cell-cell interactions being the same as *in vivo*, however, to pinpoint a certain cell type to a function or a response, it is useful to isolate individual cells or to be able to use an appropriate cell-line. Other trophoblast cells lines which could be used to investigate trophoblast cell function, and extrapolate the findings to parturition include JAR and BeWo cells (Johansen et al 2000).

An amnion cell-line, WISH, is useful for *in vitro* study of amnion pathophysiology (Pavan et al 2003). Prostaglandin and cytokine production in these cells have been characterised (Harris et al 1988, Keelan et al 1997), and prostaglandin stimulation of cytokines and its postulated role in promoting membrane rupture has been examined (Keelan et al 2000). It was also planned to test the hypothesis of this thesis in WISH cells. However, serious doubt was expressed about the origin of the cells, and they were revealed to actually be HeLa cells (Kniss et al 2002).

6.4 Relevance of the Findings to Other Biological Systems

The prostaglandin stimulation of MMP production reported in this thesis is a mechanism which occurs in many cell types over a wide range of biological systems. Progression and metastasis of cancer is one of those areas into which a lot of research has been carried out recently. COX-2 up-regulates MMP-2 production, increasing tumour cell proliferation in renal cell carcinomas (Miyata et al 2003). PGE_2 also up-regulates the

production of MMP-2 in rhabdomyosarcomas. Using selective and non-selective inhibitors of COX-2 can inhibit the progression of this cancer through reducing MMP-2 production (Ito et al 2004). COX-2 inhibition has also been shown to suppress tumour cell growth and invasion in colorectal cancer, by inhibiting MMP-2 and MMP-9 production. This also inhibited the formation of liver metastasis (Yao et al 2004). A cooperative inhibition of MMP-2 and COX-2 has been shown to reduce colon adenomas, and the cooperative inhibition allows a lower does of each inhibitor to be used, reducing the side effects of such treatments (Wagenaar-Miller et al 2003). Cooperative COX-2 and MMP-2 inhibition has also been shown treat many solid tumours, such as osteosarcomas (Dickens & Cripe 2003). Another disease in which prostaglandin stimulation of MMP production plays a role is periodontal disease. PGE_2 has been shown to mediate IL-1β induced synthesis of MMP-1 (Sakaki et al 2004) and MMP-3 (Ruwanpura et al 2004) in gingival fibroblasts. Furthermore, higher concentrations of PGE_2 and MMP-9 have been detected in gingival crevicular fluid from Down syndrome compared to control patients exhibiting gingival infection (Tsilingaridis et al 2003). In myocardial infarction and ischemic stroke, digestion by MMPs causes rupture of atherosclerotic plaques. Production of macrophage MMP-2 and MMP-9 is induced by COX-2 and PGE_2 synthesis. A polymorphism in the COX-2 gene is associated with a decreased risk of myocardial infarction and ischemic stroke, by reducing COX-2 expression, and production of MMP-2 and MMP-9 (Cipollone et al 2004). IL-1β has also been shown to induce MMP-2 via a PGE_2-dependent mechanism in human chondrocytes. This causes degradation of collagen in cartilage (Choi et al 2004).

6.5 Suggestions for Future Study

These studies have demonstrated the immunolocalisation of EP2, EP4, FP and PGDH within fetal membranes, decidua, placenta, and myometrium. It would be useful to validate this data by using Western blotting, *in situ* hybridisation and PCR. It would also be interesting to examine other prostaglandin receptors, such as EP1 and EP3, as PGE_2

is also likely to be mediating its effects through these receptors as well as EP2 and EP4. It would also be valuable to look at localisation of enzymes involved in prostaglandin synthesis, such as COX-1, COX-2 and PGES alongside the studies of PGDH. Quantatative PCR could also have been used to examine the effects of prostaglandins on the mRNA levels of the prostaglandin receptors, to establish if any positive or negative feedback cascades are being activated by addition of these prostaglandins.

Intracellular signalling via EP2, EP4 and FP has been investigated in fetal membranes and JEG3 cells. To investigate the actions upon receptor activation in the myometrium would be interesting to assess the various relaxant and stimulatory effects of prostaglandins in this tissue. Using preterm and term tissue would also be useful, as would comparing tissue from subjects in labour and not in labour to see if any differences occur across these groups. For example, non-labouring samples may act to increase more relaxant intracellular pathways, as discussed in chapter 3, to maintain uterine quiescence. In this thesis, the effect of $PGF_{2\alpha}$ on the IP_3 pathway was analysed in JEG3 cells. No effect was observed, but it would be interesting to look at this pathway in the human fetal membranes also to see if any stimulation is occurring in these. Further clarification of the action of PGE_2 via its receptors using antagonists for EP1 and EP3 receptors, alongside the antagonists already used would give more of an insight into how the cAMP and ERK pathways are stimulated. Also, the way in which the prostaglandins stimulate the ERK pathway remains unclear. Whether this is happening by transactivation of a RTK other than EGFR, or by a non-receptor tyrosine kinase remains to be discovered.

The effects of PGE_2 and $PGF_{2\alpha}$ on MMP-2, MMP-9, and TIMPs were investigated in term non-labouring human fetal membranes and placenta, and also in JEG3 cells. Comparing preterm tissue with this term tissue, and also tissue from patients in labour would be interesting to see if these regulatory factors are different. It would also be interesting to link these studies to the intracellular pathways activated by the prostaglandins. This could be carried out by stimulating the cells and tissue with a

cAMP analogue, such as dibutyryl cAMP, or ERK and investigating any changes in MMP production or activation. Also, the IL-6 effect on MMP-2 was examined in JEG3 cells. It would be relevant to examine the effect of IL-6 and also other cytokines on MMPs. It could be that cytokine stimulation of MMP production occurs via a stimulation of prostaglandins, so prostaglandin assays could be carried out to test this hypothesis.

As discussed previously in this chapter, it would also be useful to carry out these experiments in isolated chorion trophoblasts and amnion epithelial cells to further elucidate the cell types involved in regulation of prostaglandin action and MMP production and activation.

The MMPs investigated in this thesis were MMP-2 and MMP-9. Other MMPs are involved in the process of parturition (see section 5.4) and these could be studied. For example, the prostaglandin effect on the collagenases, such as MMP-1 and MMP-8 could be interesting, as these degrade many of the constituents of fetal membranes. MMP-7 also targets collagen IV, which is a major component of basement membrane in the amnion and chorion.

6.6 Summary

In summary, this study has established some of the actions of PGE_2 and $PGF_{2\alpha}$, their intracellular signalling systems, and links to tissue remodelling by MMPs at the feto-maternal interface at the time of labour. It has pointed the way to several other areas for further study, which may have some therapeutic potential for controlling the onset or progression of labour.

Appendices

Appendix 1: Buffer Compositions

1.1 Tissue and Cell Culture Buffers

1.1.1 Neutral-Buffered Formalin

Component	Concentration
Na_2HPO_4	50 mM
$NaH_2PO_4.2H_2O$	25 mM
formaldehyde	10 %

1.1.2 Steeping Buffer

DPBS containing 80 mg/l gentamycin and 5 mg/l amphotericin B

1.1.3 Complete serum-free RPMI

To a 500 ml bottle of RPMI-1600, remove 10 ml and add 5 ml penstrep (5000 IU/ml penicillin and 5 mg/ml streptomycin) and 5 ml growth factors (5 μg/ml insulin, 5 μg/ml transferring, 5 ng/ml sodium selenite).

1.1.4 Complete DMEM

With serum: To a 500 ml bottle of DMEM, remove 65 ml and add 50 ml fetal calf serum, 10 ml L-glutamine (200 mM stock) and 5 ml penstrep.

Serum-free: To a 500 ml bottle of DMEM, remove 15 ml and add 10 ml L-glutamine (200 mM stock) and 5 ml penstrep.

1.1.5 Trypan Blue

0.5 mg/ml trypan blue in 0.85 % NaCl

1.2 Immunohistochemistry Buffers

1.2.1 PBS wash buffer (pH 7.5)

Component	Concentration
$NaH_2PO_4.H_2O$	0.2 g/l
Na_2HPO_4	1.375 g/l
NaCl	8.8 g/l

1.2.2 Scott's Solution

Component	Concentration
$NaHCO_3$	3.5 g/l
$MgSO_4$	20 g/l

1.2.3 Blocking Solution

10 ml TBS, 0.5 g BSA, 3 drops goat serum (from ABC kit)

Use to dilute primary and secondary antibodies

1.2.4 ABC-HRP

Use from kit. Make up 20 min in advance.

Dilute stock Tris (see TBS wash buffer, 1.2.7) to 50 mM and add 1 drop reagent A and 1 drop reagent B

1.2.5 DAB

Use from kit (Vector). To 5 ml distilled water, add 2 drops buffer stock solution, 4 drops DAB stock and 2 drops of H_2O_2 stock

1.2.6 Citrate Buffer (x10)

0.1 M citric acid (monohydrate) in water

Adjust to pH 5.5 using NaOH, dilute 1 in 10 for use

1.2.7 TBS wash buffer

Make stock 1M Tris. Adjust to pH 7.4 using 5N HCl

For use, add NaCl to 8.5 g/l

1.3 PCR Buffers

TAE Buffer (x50)

Component	Concentration
Tris Base	242g/l
Glacial acetic acid	57.1 ml/l
0.5 M EDTA pH8.0	100 ml/l

Dilute 1 in 50 for use

1.4 Assay Buffers

1.4.1 Protein Assay

1.4.1.1 0.2 N Sodium Hydroxide

4 g NaOH in 500 ml water

1.4.1.2 3 % w/v Sodium carbonate

15 g Na_2CO_3 in 500 ml 0.2 M NaOH

1.4.1.3 4 % w/v Potassium Sodium Tartrate

4g KNa Tartrate 100 in ml water

1.4.1.4 2 % w/v Copper Sulphate-5-Hydrate

2 g $CuSO_4.5H_2O$ in 100 ml water

1.4.1.5 Solution A

Component	Volume /25ml
3 % Na_2CO_3	24 ml
4 % KNa Tartrate	0.5 ml
2 % Copper Sulphate-5-Hydrate	0.5 ml
'Reagent S'	105 µl

1.4.1.6 Folin and Ciocalteu's Phenol Reagent

Dilute solution 1:1 with water

1.4.2 Prostaglandin Assay

1.4.2.1 Methyloximating Solution

Component	Concentration
Na acetate	1 M
Methoxyamine HCl	0.1 M
ethanol	10 % v/v

Adjust to pH 5.6 with 5N HCl

1.4.2.2 Dry Coat Solution

Component	Concentration
Polyvinyl pyrollidone	2 %
BSA	5 mg/ml
EDTA	5 mM
Tris-HCl	50 mM
2-methylisothiazolone (in 1:1 DMF/DMSO)	1 mM
Bromonitro dioxane (in 1:1 DMF/DMSO)	1 mM

1.4.2.3 PG assay wash buffer

Component	Concentration
NaCl	150 mM
Tris-HCl	10 mM
Tween-20	0.05 % v/v

pH 7-7.5

1.4.2.4 ELISA buffer +/- Tween

Component	Concentration
NaCl	150 mM
Tris-HCl pH7.2	100 mM
Phenol red solution	50 mM
EDTA	2 mM
2-methylisothiazolone	1 mM
bromonitrodioxane	1mM
BSA	2 mg/ml
Tween-20	0.05 % v/v

1.4.2.5 25 % MOX buffer

25 % Methyloximating solution in ELISA buffer with Tween

1.4.2.6 Phosphate buffer

0.5 M $Na_2H_2PO_4.H_20$ pH 8.0

1.4.2.7 TMB substrate

Component	Volume for 24 ml
100 mM Sodium acetate pH 6.0	20 ml
Tetramethylbenzidine in DMF	2 ml
Urea-peroxidase 0.3 g/50 ml in 100 mM sodium acetate pH 6.0	2 ml

1.4.3 IP_3 Assay- Buffer A

Component	Concentration
NaCl	140 mM
HEPES	20 mM
KCl	4 mM
glucose	8 mM
$MgCl_2$	1 mM
$CaCl_2$	1mM
BSA	1 mg/ml

1.5 Electrophoresis Buffers

1.5.1 Western Blot

1.5.1.1 Homogenisation/Extraction Buffer

Component	Concentration
TrisHCl	20 mM
NaCl	150 mM
SDS	0.1 % w/v
Protease inhibitor tablet	1 tablet/ 50 ml

Adjust to pH 7.4 with 5N HCl

1.5.1.2 NP40 Lysis Buffer

Component	Concentration
NaCl	150 mM
Tris pH 7.4	50 mM
EDTA	10 mM
EGTA	10mM
NP40	0.6 % v/v
Glycerol	10 % v/v
Protease inhibitor tablet	1 tablet/ 10 ml

1.5.1.3 Sample application buffer

Component	Concentration
Tris	1.5 % w/v
SDS	4.0 % w/v
Dithiothretiol (DTT)	2.0 % w/v
Bromophenol blue	0.05 % w/v

Adjust to pH 6.75 with 5N HCl, store –20 °C

1.5.1.4 Running tank buffer (x10)

Component	Concentration
Tris	0.25 M
glycine	1.9 M
SDS	1.0 % w/v

1.5.1.5 Stock Wash Buffer TBS (x10)

Component	Concentration
Tris	25 mM
glycine	192 mM
methanol	20 % v/v

pH 8.1-8.4

1.5.1.6 Transfer buffer

Component	Concentration
Tris	0.5 M
NaCl	1.5 M

Adjust to pH 7.4 with 5N HCl

1.5.1.7 TTBS

Tween 20 0.05 % v/v in TBS x1

1.5.1.8 Membrane Blocking buffer

BSA 5 % w/v in TTBS

1.5.1.9 Stripping solution

Component	Concentration
glycine	0.2 M
SDS	1 % w/v

Adjust to pH 2.5 with 5N HCl

1.5.2 Gelatin Zymography/Reverse Zymography

1.5.2.1 Sample Application Buffer

Component	Concentration
Glycerol	20 % w/v
SDS	2.0 % w/v
Bromophenol blue	0.04 % w/v

1.5.2.2 Running tank buffer (x10)

Component	Concentration
Tris	0.25 M
glycine	1.9 M
SDS	1.0 %

1.5.2.3 Gelatin Zymography Wash buffer TBS (x10)

Component	Concentration
Tris	0.5 M
NaCl	1.5 M

Adjust to pH 8.0 with 5N HCl

1.5.2.4 Reverse Zymography Wash buffer

Component	Concentration
Tris	50 mM
$CaCl_2$	5 mM
Triton-X-100	2.5 % v/v

Adjust to pH 7.5 with 5N HCl

1.5.2.5 Gelatin Zymography Triton X-100 wash

2.5 % Triton X-100 in TBS 1:10

1.5.2.6 Gelatin Zymography Digestion buffer

Component	Concentration
Tris	50 mM
NaCl	0.2 M
$CaCl_2$	5.0 mM
$ZnCl_2$	1.0 μM
Brij-35	0.02 % v/v

Adjust to pH 7.6 with 5N HCl

1.5.2.7 Reverse Zymography Digestion Buffer

Component	Concentration
Tris	50 mM
$CaCl_2$	5 mM

Adjust to pH 7.5 with 5N HCl

1.5.2.8 Destaining solution

Component	Concentration
methanol	30 %
Glacial acetic acid	10 %
water	60 %

1.5.2.9 Staining solution

0.5 % w/v Coomassie Brilliant Blue R250 in destaining solution

Appendix 2: Gel compositions

2.1 Western Blot

2.1.1 Resolving gel (for 2 gels 1.5 mm thick)

Component	Volume (for 10% gel)	Volume (for 12 % gel)
ddH_20	8.1 ml	6.7 ml
Tris 1.5 M pH 8.8	5.0 ml	5.0 ml
10 % SDS	200 µl	100 µl
10 % ammonium persulphate	100 µl	100 µl
Acrylamide/Bis	6.6 ml	8.0 ml
TEMED	10 µl	10 µl

Before addition of TEMED, mixture was de-gassed via water pressure/dessicator vacuum for 10 min

2.1.2 Stacking gel 4 % (for 2 gels 1.5 mm thick)

Component	Volume
ddH_20	6.1 ml
Tris 0.5 M pH 6.8	2.5 ml
10 % SDS	100 µl
10 % ammonium persulphate	100 µl
Acrylamide/Bis	1.3 ml
TEMED	10 µl

2.2 Gelatin Zymography

2.2.1 Resolving gel 7.5 % (for 2 gels 0.75 mm thick)

Component	Volume
ddH_20	3.85 ml
Tris 1.5 M pH 8.8	2.5 ml
Gelatin 10 mg/ml	1.0 ml
10 % SDS	100 µl
10 % ammonium persulphate	50 µl
Acrylamide/Bis	2.5 ml
TEMED	5 µl

NB. Gelatin was dissolved in the microwave prior to addition.

2.2.2 Stacking gel 4 % (for 2 gels 0.75 mm thick)

Component	Volume
ddH_20	3.05 ml
Tris 0.5 M pH 6.8	1.25 ml
10 % SDS	50 µl
10 % ammonium persulphate	50 µl
Acrylamide/Bis	0.65 ml
TEMED	5 µl

2.3 Reverse Zymography

2.3.1 Resolving gel 12 % (for 2 gels 0.75 mm thick))

Component	Volume
ddH_20	1.69 ml
Tris 1.5 M pH 8.8	2.5 ml
Gelatin 10 mg/ml	1.0 ml
10 % SDS	100 µl
10 % ammonium persulphate	50 µl
Acrylamide/Bis	4.0 ml
Solution A	0.66 ml
TEMED	5 µl

2.3.2 Stacking gel 5 % (for 2 gels 0.75 mm thick)

Component	Volume
ddH_20	2.89 ml
Tris 0.5 M pH 6.8	1.25 ml
10 % SDS	50 µl
10 % ammonium persulphate	50 µl
Acrylamide/Bis	0.812 ml
TEMED	5 µl

2.4 PCR Product Gels

1.8 g agarose in 120 ml TAE buffer (appendix 1.3).

Dissolve in microwave, then add 6 µl ethidium bromide and pour.

Appendix 3: Materials

The source of all materials used for all experiments is detailed in the table below. They have been sorted into sections according to the methods used. Unless otherwise stated, all acids, solvents and salts were purchased from BDH, Lutterworth, UK, and all cell culture sterile plasticware from Corning Life Sciences, NY, USA.

Materials – Tissue/Cell culture	Source
formaldehyde	Sigma, Poole, UK
paraffin	Bayer Diagnostics, Newbury, UK
DPBS	Gibco, Paisley, UK
heparin	LeoLabs Ltd., Risborough, UK
gentamycin	Sigma, Poole, UK
Amphotericin B	Sigma, Poole, UK
RPMI	Gibco, Paisley, UK
penstrep	Gibco, Paisley, UK
Growth factors	Sigma, Poole, UK
Capillary matting	B&Q Edinburgh, UK
JEG3 cell-line	European Collection of Cell Cultures (ECACC), Salisbury, UK
DMEM	Gibco, Paisley, UK
Fetal calf serum	Gibco, Paisley, UK
L-glutamine	Gibco, Paisley, UK
Trypsin/EDTA	Gibco, Paisley, UK
Trypan blue	BDH, Lutterworth, UK
Indomethacin	Sigma, Poole, UK
IBMX	Sigma, Poole, UK
PGE_2	Upjohn, Kalamazoo, MI, USA
$PGF_{2\alpha}$	Upjohn, Kalamazoo, MI, USA

DMSO	Sigma, Poole, UK
PD98059 (MEK inhibitor)	Calbiochem, Nottingham, UK
U73122 (PLC inhibitor)	Calbiochem, Nottingham, UK
AG1478 (EGFR kinase inhibitor)	Calbiochem, Nottingham, UK
AH6809 (EP2 antagonist)	Cayman Chemicals, Ann Arbor, Michigan
ONO-AE2-227 (EP4 antagonist)	Dr. Henry Jabbour, HRSU, MRC
AL8810 (FP antagonist)	Sigma, Poole, UK
IL-6	Peprotech, London, UK

Materials -Immunohistochemistry	**Source**
AAS	Sigma, Poole, UK
BSA	Sigma, Poole, UK
Hydrogen peroxide	Sigma, Poole, UK
Triton-X-100	BDH, Lutterworth, UK
Cytokeratin 1° Ab (#Z622)	Dako Corp, Santa Barbara, CA, USA
EP2 1° Ab (#101750)	Cayman Chemicals, Ann Arbor, Michigan
EP4 1° Ab (#101775)	Cayman Chemicals, Ann Arbor, Michigan
FP 1° Ab (#101802)	Cayman Chemicals, Ann Arbor, Michigan
PGDH 1° Ab (Tai et al 1990)	Prof H.H. Tai, Lexington, Kentucky, USA
ABC Vectastain Kit	Vector Laboratories, Peterborough, UK
DAB Vactastain Kit	Vector Laboratories, Peterborough, UK
haematoxylin	Sigma, Poole, UK
Eosin	BDH, Lutterworth, UK
Pertex	Cell Path, Hemel Hempstead, UK

Materials - PCR	Source
Tri-reagent	Sigma, Poole, UK
Chloroform	Sigma, Poole, UK
Isopropanol	Sigma, Poole, UK
Nuclease free water	Promega, Madison, USA
DNase treatment reagents	Promega, Madison, USA
RT-reaction reagents	Applied Biosystems, Foster City, CA
PCR primers	MWG Biotech, Ebersberg, Germany
RT-PCR reagents	Promega, Madison, USA
100 bp DNA ladder	Promega, Madison, USA
Blue/Orange 6x loading dye	Promega, Madison, USA
Lightcycler mix	Roche Diagnostics, Mannheim, Germany

Materials - Assays	Source
BSA	Sigma, Poole, UK
Reagent 'S'	Biorad, Hemel Hempstead, UK
F& C Reagent	BDH, Lutterworth, UK
Tween-20	Sigma, Poole, UK
Phenol Red	Sigma, Poole, UK
Polyvinyl pyrollidone	Sigma, Poole, UK
2-methylisothiazolone	Boehringer Mannheim, Lewes, UK
bromonitrodioxane	Boehringer Mannheim, Lewes, UK
PGE_2 antibody	Diagnostics Scotland, Edinburgh, UK
Antiserum	Prof. Rodney Kelly, HRSU, MRC
PGE_2 assay link	Prof Rodney Kelly, HRSU, MRC
Streptavidin peroxidase	Boehringer Mannheim, Lewes, UK
Hydrogen Peroxide Urea Complex	Sigma, Poole, UK
Tetramethyl benzidine in DMF	Sigma, Poole, UK
cAMP assay kit (#RPN225)	Amersham, Buckinghamshire, UK

Dowex resin	Biorad, Hemel Hempstead, UK

Materials - Electrophoresis	**Source**
Dialysis tubing	Sigma, Poole, UK
Protease inhibitor tablets	Roche Diagnostics, Mannheim, Germany
NP40	Calbiochem, Nottingham, UK
Glycerol	BDH, Lutterworth, UK
DTT	Sigma, Poole, UK
Bromophenol blue	Biorad, Hemel Hempstead, UK
Tween-20	Sigma, Poole, UK
BSA	Sigma, Poole, UK
Triton-X-100	BDH, Lutterworth, UK
Brij-35 solution	Sigma, Poole, UK
Coomassie Brilliant Blue R250	Biorad, Hemel Hempstead, UK
Glacial acetic acid	Sigma, Poole, UK
Ammonium persulphate	Sigma, Poole, UK
Acrylamide	Biorad, Hemel Hempstead, UK
TEMED	Sigma, Poole, UK
Gelatin (Bovine #G-9382)	Sigma, Poole, UK
Sec-butanol	Sigma, Poole, UK
Zinc chloride	Sigma, Poole, UK
Molecular weight markers	Biorad, Hemel Hempstead, UK
ECL reagents	Amersham, Buckinghamshire, UK
Kodak film	Sigma, Poole, UK
Reverse Zymography Solution 'A'	Dylan Edwards, UEA, Norwich
Agarose	Sigma, Poole, UK
Ethidium bromide	Sigma, Poole, UK
P42/44 phospho ERK 1° Ab (#9101(S))	Cell Signalling Technology
MMP-2 1° Ab (#1M33L)	Calbiochem, Nottingham, UK

MMP-9 1° Ab (#1B7013)	Insight Biotechnology, Wembley, UK
TIMP-1 1° Ab (#T7562)	Sigma, Poole, UK
TIMP-2 1° Ab (#AB19078)	Chemicon, Harrow, UK
TIMP-3 1° Ab (#T7812)	Sigma, Poole, UK
TIMP-4 1° Ab (#AB816)	Chemicon, Harrow, UK
DAR 2° Ab (#NA934)	Amersham, Buckinghamshire, UK
SAM 2° Ab (#NA931)	Amersham, Buckinghamshire, UK

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